AN INVESTIGATION INTO THE DISTRIBUTION AND PROPERTIES OF BASIC PROTEINS (HISTONES) IN MICRO-ORGANISMS

A thesis presented for the Degree

of

Doctor of Philosophy

by

John Livsey Leaver

Department of Biochemistry,
University of Edinburgh.

October, 1964.

TABLE OF CONTENTS

Table of Contents (contd.) Page

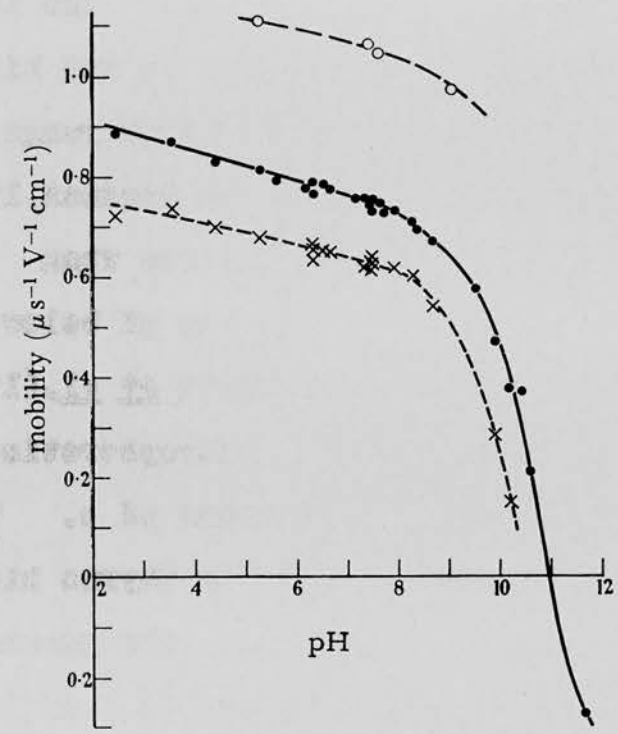
Table of Contents (contd.) Page

INTRODUCTION

Little is known about the distribution of basic proteins in micro-organisms, even although it has been suggested that the histones and protamines, which occur in close association with deoxyribonucleic acid (D.N.A.) in the nuclei of vertebrate animals, might be involved in the expression or replication of the genetic material. Because of the variety of different basic proteins which are now known to exist, it is necessary to consider what is understood by the terms "histone" and "protamine".

The occurrence of the basic compound protamine in salmon spermatozoa was first reported in 1874 by Miescher, after his classical researches into the chemical nature of cell nuclei. This was followed by the isolation of histone from goose erythrocyte nuclei by Kossel in 1884. Histone was later found to be present in calf thymus glands (Lilienfield, 1894), in fowl erythrocyte nuclei (Ackerman, 1904), and in place of protamine in the spermatozoa of some fish (Kossel, 1928).

Figure 1. Mobility-pH graph of histones from ox thymus nuclei. ○, α-component. ●, β-component. ×, γ-component.
Taken from Cruft et al. (1957)



The analogy between these two basic compounds, proposed by Kossel, and their relationship to the proteins in general, were only confirmed some years later after the analysis of their hydrolysis products (Kossel, 1928).

The most characteristic property of the histones is their very basic nature. The iso-electric points of different histones and histone fractions occur normally within the pH range of 10.0 to 11.0 (Cruft, Mauritzen and Stedman, 1957 a), and thus they will migrate as cations when subjected to electrophoresis at any pH below 10. Using the Tiselius apparatus, Cruft et al.(1957 a) made a detailed study of the electrophoretic mobilities of histones at different pH s. The mobility-pH curves for the main ox thymus histone components are shown in Fig. 1. The general shape of these curves, and the marked change in the gradients at about pH 9, are characteristic of most histones; only histones isolated ffom certain tumour tissues were found to exhibit rather unusual mobility-pH curves.

TABLE 1

Amino acid analyses of total histone and histone fractions of calf thymus, and of salmine and clupein

Amino acid residue	Total histone (ref. 1)	β fraction (ref. 2)	1.6s α fraction (ref. 3)	α_4 fraction (ref. 4)
Arginine	10.3	16.1	11.2	0
Lysine	15.5	9.9	12.5	49.7
Histidine	2.2	2.7	2.9	0
Aspartic acid	4.4	5.7	6.3	0.3
Glutamic acid	8.5	10.7	9.9	0.8
Glycine	3.9	4.8	4.9	2.1
Alanine	7.7	7.7	8.1	22.5
Valine	4.9	5.1	5.7	3.1
Leucine	7.1	9.9	9.5	0.7
Isoleucine	4.0	4.9	4.9	0
Phenylalanine	2.5	2.4	1.4	0
Tyrosine	3.3	3.8	4.4	0
Serine	4.1	3.0	5.4	2.3
Threonine	4.6	5.1	4.6	4.4
Proline	3.9	3.3	3.1	14.1
Methionine	1.0	1.4	1.1	0

(all values as g. amino acid residue per 100 g. protein)

Table 1 (contd.)

Amino Acid residue	α_2 fraction (ref. 4)	α_3 fraction (ref. 4)	salmine (ref. 5)	clupein (ref. 5)
Arginine	6.8	6.7	80.4	73.6
Lysine	34.7	24.0	0	0
Histidine	0.4	1.6	0	0
Aspartic acid	2.9	4.9	0	0
Glutamic acid	5.5	6.4	0	0
Glycine	5.3	5.9	1.6	0.3
Alanine	16.7	12.8	0.3	3.8
Valine	4.8	5.7	1.4	3.6
Leucine	5.1	8.3	0	0
Isoleucine	1.2	2.2	0.3	0.7
Phenylalanine	0.8	1.7	0	0
Tyrosine	1.0	2.7	0	0
Serine	4.5	5.9	2.7	4.3
Threonine	5.4	4.3	0	2.0
Proline	9.8	6.2	2.5	5.2
Methionine	0	0.6	0	0

References:
1. Data of Crampton, et al. (1955) converted to g. amino acid residue per 100 g. protein.
2. Data of Cruft et al. (1958 a).
3. Data of Cruft et al. (1958 b).
4. Data of Cruft et al. (1957 b) converted to g. amino acid residue per 100 g. protein.
5. Data of Ando et al. (1959) converted to g. amino acid residue per 100 g. protein.

The amino acid analyses of unfractionated calf thymus histone and some of its fractions are given in Table 1. The analyses of two protamines have also been included for comparison. This table demonstrates the wide variations which occur in the composition of different histone fractions, and illustrates why it is impossible to define the term "histone" solely on the basis of amino acid composition. However the amino acid analyses of histones do have certain characteristic features. There is always a considerable excess of basic over acidic amino acids, with lysine and arginine together accounting for 25% (w/w), or more, of all the amino acid residues. Histones also usually contain relatively large amounts of alanine, leucine, and the two acidic amino acids. Apart from the predominance of lysine and arginine, the most notable feature of the composition of histones is the absence of tryptophan and cysteine. Traces of cysteine have occasionally been reported, normally in unfractionated histone preparations (Phillips, 1962), but this may be due to contamination with other proteins.

Because of the absence of tryptophan, the ultra-violet absorption spectra of the histones are, in general, very like those of tyrosine (Cruft, 1953). Cruft has used the spectra both to show that preparations are free from contamination with nucleic acids, and to determine the approximate tyrosine content of histones.

The molecular weights reported for different calf thymus histone fractions vary from a few thousand to almost 100,000 (reviewed by Phillips, 1962). This demonstrates, along with the amino acid analyses, the wide variety of different molecules which must be accommodated by the term "histone". The precise definition of a histone, even from a well studied source such as calf thymus, is therefore most difficult. This great diversity of the histones, and even of histone sub-fractions, is particularly well demonstrated by zone electrophoresis in starch and polyacrylamide gels (Neelin and Neelin, 1960; Muecke, 1962; Cruft, 1964).

In view of earlier definitions of the term "histone", which had led some authors to classify the weakly basic protein globin under this heading, Cruft et al. proposed in 1957 that the term "should be reserved exclusively for basic proteins, other than protamines, which occur in the cell nucleus".

Since then basic proteins, with amino acid analyses similar to those of the histones, have been isolated from rat liver microsomes (Butler, Cohn and Simson, 1960), and starch gel electrophoresis of acid soluble proteins extracted from ribosomes of pea seedling buds has revealed several protein components with isoelectric points above pH 7 (Setterfield, Neelin, Neelin and Bayley, 1960). Leslie (1961) has also shown that basic proteins extracted from ribosomes of cultured human liver cells (strain HLM) are similar in composition to the histones.

The existence of these cytoplasmic histone-like proteins may eventually necessitate some alterations in the nomenclature. Phillips (1962) suggests that if these cytoplasmic basic proteins are related metabolically with the histones, then the term histone could be widened to include them; however, if they are not directly connected with the histones, but possess some function with the ribonucleic acids similar to that of the histones with the deoxyribonucleic acids, then the term cytoplasmic histones would be appropriate.

No metabolic or functional relationship has yet been established between these two classes of protein, and therefore in this dissertation the cytoplasmic basic proteins have been simply named by their origin in the cell. For instance, basic proteins extracted from ribosomes are referred to as "ribosomal basic proteins". The term histone has been reserved for basic proteins of nuclear origin, or, in organisms which have no well defined nucleus, for the basic proteins occurring in association with DNA.

Recently, at the First World Conference on Histone Biology and Chemistry, Murray (1964) suggested that histones should be defined as "basic proteins that at some time are associated with DNA". Murray justified this broadening of the definition to include protamines on the basis that the histones and protamines are homologous proteins, and that their distinction is little different from that between two histone fractions of widely different properties.

The literature providing evidence for the wide distribution of histones and protamines in multi-cellular organisms is summarised in the Appendix. This is an adapted and extended version of a similar summary given by Phillips, (1962) in a review.

The presence of histones has been demonstrated in a variety of tissues isolated from many different vertebrate species, and it is now generally accepted that they are of universal occurrence in the somatic cell nuclei of all vertebrates, (Cruft, et al. 1957 a; Mirsky and Osawa, 1961; Phillips, 1962). Although histones are present in the spermatozoa of some vertebrates the simpler protamines occur in the spermatozoa of certain fish and the chicken.

No extensive studies have been carried out into the distribution of basic proteins in the invertebrate animals. However, in the snail Helix aspersa, cytological techniques have indicated the existence of histones in the ovum and developing embryo, and of protamines in the spermatozoa. Basic proteins similar in nature to histone have also been isolated from the spermatozoa of several echinoderms and from the gills and testis of the squid.

Although histones have been isolated from relatively few plant species, their presence in several others have been inferred from cytological staining, and it therefore appears probable that they also occur widely throughout this group of organisms.

In the field of micro-organisms little is known about the distribution of histones. Indeed at the time this investigation was commenced, most of the available evidence favoured the view that histones were absent from bacteria.

In 1947 Belozerskii had reviewed his work on the properties of nucleoproteins prepared from various bacterial species. His isolation procedure involved the extraction of the bacteria with dilute alkali and precipitating the nucleoprotein with acid. On the basis of analyses of the basic amino acids present in his preparations, he concluded that proteins of basic character are absent in bacterial nucleoproteins. However, it could be argued that the alkaline extraction and acid precipitation of nucleoproteins would favour the recovery of acidic proteins along with the DNA, at the expense of any basic proteins present.

The failure of Chargaff and Saidel (1949) to detect histones in a preparation of nucleoprotein from avian tubercle bacilli is open to the same criticisms.. Their isolation involved extraction with pH 8.3 borate buffer, and precipitation with acetic acid at pH 4.2.

More recently deoxyribonucleoproteins have been isolated from other species of bacteria (Tsumita and Chargaff, 1958; Masui, Iwata, Ishimitsu and Umebayashi, 1962) using methods based on that of Chargaff and Saidel. Amino acid analyses of the preparations again led to the conclusion that the protein moieties were non-basic.

In agreement with these authors, Belozerskii and Uryson (1958) were unable to isolate histones from either the nucleoproteins of Proteus vulgaris or by direct extraction of the bacterial mass.

DNA-protein was prepared from Escherichia coli by Zubay and Watson (1959) using a technique similar to one commonly used for the isolation of DNA-histone from higher organisms. This involved extraction with water followed by precipitation of the DNA-protein with pH 8 buffered 0.15M sodium

chloride. Analysis of the amino acids liberated on the hydrolysis of this preparation, together with studies of its X-ray diffraction pattern (Wilkins and Zubay, 1959), led to the conclusion that E. coli do not contain any histone. However, these authors do point out that it is not certain whether the protein which they have studied is naturally bound to DNA in the bacterial cell, or becomes attached during isolation. Indeed the X-ray diffraction study showed that a large proportion of the DNA in the DNA-protein preparation is free of protein, and Wilkins and Zubay believe that this indicates that most of the DNA in the intact bacteria is not attached to protein.

Observations with the electron microscope by Van Iterson and Robinow (1961) on the fine structure of nuclear bodies in two spherical bacteria, demonstrated the presence of fibers with a diameter of 20 Ångstom units. This diameter is close to that of the DNA molecule in the model of Watson and Crick (1953) and it was therefore concluded, in agreement with Wilkins and Zubay, that most of the length of the DNA molecules in bacteria is not associated with protein.

In 1958 Spiegelman, Aronson and Fitz-James isolated nuclear bodies from protoplasts of Bacillus megaterium. The protein content of the nuclear bodies was determined by two methods; the ninhydrin method of Moore and Stein (1958), and the Folin procedure (Lowry, Rosebrough, Farr and Randall, 1951). As the purification of the nuclear bodies progressed, the ratio of the ninhydrin value to the Folin value increased. Since histones give a relatively weak Folin colour and a strong ninhydrin colour, this was taken as indicating that the megaterium structures contain proteins analogous to the histones. However, this is a poor method for characterising the presence of histones, and, moreover, Butler and Godson (1963) have failed to extract basic protein from a similar nuclear fraction of B. megaterium.

At the time this investigation was started, only one paper, that of Palmade, Chevallier, Knobloch and Vendrely (1958), provided any real evidence for the possibility that histones might be present in bacteria. These authors stated that the difficulty in extracting good preparations of DNA-protein from bacteria was probably

due to their rigid cell walls. They obviated this difficulty by preparing protoplasts from E. coli, and isolated DNA-protein by the classical method of molar salt extraction, followed by precipitation at physiological salt concentration. Analysis of their DNA-protein gave a nitrogen to phosphorus ratio which was almost identical to that of a typical nucleohistone. The amino acids liberated on hydrolysis of their product were studied by paper chromatography, and the pattern and intensities of the ninhydrin developed spots were similar to those obtained from a hydrolysate of trout erythrocyte nucleohistone.

More recently this work was extended by Palmade (1961), who studied the ratios of arginine and lysine to total protein in DNA-proteins prepared from E. coli and Micrococcus Lysodeikticus. It was concluded that typical nucleohistones are present in these organisms. However, it should be emphasised that this author does not state the yield of DNA-protein obtained; also, that in the best preparations, DNA and protein together only accounted for some 70% of the material present, the contaminant being nitrogenous in nature, and that no attempt was made to actually isolated histone by acid extraction of the DNA-protein.

If DNA in the bacterial cell is largely complexed with histone, as in the nuclei of higher organisms, it is surprising that any great difficulty should be encountered in obtaining good yields of DNA-histone. Most bacteria appear to contain DNA in the order of 1 to 5% of their dry mass (Handbook of Biological Data, 1956). If this DNA were largely combined with histone, then the bacteria would contain between 2 and 10% nucleohistone.

The conflicting reports, relating to the possible occurrence of histones in bacteria, could be reconciled to some extent if only a small proportion of the DNA in the cell were bound to basic protein. If this were so, then the yield of DNA-histone obtained by Palmade (1961) would be low. However, if histones are absent in bacteria, then the DNA-basic protein complex of Palmade might be an artefact due to combination, during the extraction procedure, of free DNA with basic proteins of some other origin in the cell, possibly ribosomal. Indeed the presence of basic proteins in E. coli ribosomes is indicated by the result of Waller and Harris (1961). The acid soluble proteins of the

ribosomes were separated into two fractions by chromatography on carboxymethyl-cellulose, and their behaviour on subjection to starch gel electrophoresis was studied. The "more basic" fraction obtained was not further characterised, and neither the amino acid composition nor the isoelectric point was determined. Zubay and Wilkins, (1960) also concluded that histone-like proteins are probably present in E. coli ribosomes after X-ray diffraction studies of their structure.

Although nearly a century has passed since the discovery of histones, and it has been established that they are quantitatively a very important component of the cell nucleus in higher organisms, accounting for some 20 to 25% of the dry weight (Cruft, et al. 1957 a), little has yet been learnt about their biological role.

It has been considered that the histones might serve a purely structural function in the cell nucleus, where it was visualised that they link the DNA molecules into a chromosome (Butler, 1956). This seems unlikely, as the morphology of the chromosomes is not destroyed by the Feulgen

staining technique, which removes the histone and possibly much of the DNA from the specimen (Stedman and Stedman, 1944 a). That nucleic acid itself is not required to preserve the structure of the chromosomes follows from the fact that chromosomes can still be stained after treatment with nuclease preparations (Mazia, 1941). Also, Mirsky (1947) has shown that the removal of most of the DNA and histone from chromosomes by extraction with molar salt leaves residual chromosomes, consisting mainly of a tryptophan containing protein. This non-basic protein, which has been given the name chromosomin by the Stedmans, would therefore appear to the main structural component of the chromosomes.

The hypothesis that histones have a passive role in neutralising the acid groups of DNA, and so prevent the DNA from associating with other proteins or metal ions, is in agreement with the reported equivalence between the basic groups of histone and the phosphate groups of DNA in native DNA-histone (Vendrely, Knobloch-Mazen and Vendrely 1960). However, such a theory appears to be rather naive as it fails to take into account the apparent diversity of histones, demonstrated by the zone electrophoresis of histone fractions in starch gel (Neelin and Neelin, 1960; Muecke,1962), or their relatively high turnover rate (reviewed by Phillips, 1962).

The first evidence for the occurrence of cell specificity in nuclear basic proteins was the demonstration by Stedman and Stedman (1944 b) that histone is present in the liver of the salmon, whereas the spermatozoa of this fish contain protamine. Later it was shown, by small differences in amino acid composition, that, in some species, specificity of histones can also occur between different somatic cell types. This led the Stedmans to postulate that one of the physiological functions of histones might be the control of cell differentiation, by acting as "gene suppressors", (Stedman and Stedman, 1950). In such a system it is visualised that the histones exert their effect by combining specifically with areas of the DNA structure not required for the production of a given type of cell, and so allowing only the uncombined DNA to determine the character of the cell.

This theory of the Stedmans is attractive as it would account for the diverse nature of the histones. Moreover, Bonner, Huang, and Gilden (1963) have recently obtained some direct evidence in its favour, by studying the chromatin dependent synthesis of a cell specific pea seedling protein.

Leslie (1961) has demonstrated ribonuclease (RNA ase) activity in histones and ribosomal basic proteins. He suggested that these proteins may exert control in cell differentiation processes by eliminating certain RNA templates, while stabilising others by combining with them to form RNA-protein complexes. The enzymic activity of his preparations was apparently rather low, and subsequent results indicate that only a small portion of the basic protein possess the RNAase activity (Martin, England, Turkington and Leslie, 1963).

If the sole function of histones is the control of cell differentiation in multicellular organisms, by either of the two postulated schemes, then their absence in bacteria and protozoa might be expected. However, if they have an even more fundamental role related to the function of the genetic material in all living cells, then their presence in unicellular organisms would be expected.

It has also been suggested that histones could be concerned with mitosis; possibly by being involved in the separation of the chromatids (Butler, 1956). In this event they would be present in all cell types which undergo mitotic division, but not necessarily in bacteria.

Such considerations regarding the possible physiological functions of histones, together with the contradictory reports concerning their occurrence in bacteria, prompted this investigation into the distribution of basic histone-like proteins in micro-organisms. In each case the first aim has been to establish, by direct extraction of the whole organisms, the existence of such proteins, and then to proceed with the study of their intracellular distribution and fuller characterisation.

EXPERIMENTAL METHODS

I. Isolation of Calf Thymus Histone

Calf thymus histone was prepared by the method of Stedman and Stedman (1951), with the later modification of Cruft, Mauritzen and Stedman (1957) in which the isolation of the thymus nuclei was carried out in the cold. This procedure will be described in some detail, as the methods developed for the isolation of basic proteins from micro-organisms have been based on it as far as practical . Calf thymus histone has also been used throughout this investigation as a standard for comparison during the characterisation of other basic proteins.

i. Isolation of Nuclei

All operations were carried out at 2°C, either in a cold room or a M.S.E. "Major" refrigerated centrifuge. The freshly excised glands were brought to the laboratory mixed with ice cubes. After washing in 4% acetic acid and removing any fat and connective tissue, the glands were passed through a coarse mincer followed by an extrusion mincer. The tissue pulp was then suspended in 2 volumes of 4% acetic acid and

stirred for 2 hr. During this time much fibrous material adhering to the stirrer blades was removed.

The mixture was filtered through four layers of butter muslin and the filtrate centrifuged for 15 min. at 1000 x g. The supernatant was discarded and the sedimented nuclei resuspended in 6 volumes of 1% acetic acid. The nuclei were washed some eight or ten times with 1% acetic acid by repeating the centrifugation and resuspension processes. Finally, when a sample was stained with methylene blue and examined under the microscope, the nuclei were seen to be free from cytoplasmic contamination.

The nuclei were then defatted and dried by washing three times each with ethanol and ether - one of the ethanol washes being carried out overnight to ensure thorough extraction of lipid material. This procedure will also denature many of the non-basic proteins, rendering them insoluble to the subsequent acid extraction. After the final wash, the ether was allowed to evaporate slowly, while the nuclei were occasionally stirred gently. When no further ether evaporation could be detected by smell, the air-dried nuclei were put in a stoppered bottle for storage.

ii. Extraction of Histone

The dry nuclei were extracted with 0.1$\bar{N}$ sulphuric acid (10 ml. per g.) for 1 hr. at room temperature. The nuclei were then sedimented by centrifugation (5000 x g for 15 min.), and the supernatant mixed with 7 volumes of ethanol to precipitate the histone sulphate. The nuclei were re-extracted with the acid until no further precipitate was obtained with ethanol - usually on the fourth extraction. The ethanol precipitates were then pooled and collected by centrifugation (5000 x g for 10 min.).

The choice of organic solvent for the precipitation of histones from acid extracts is important as histone sulphate is incompletely precipitated by acetone, whereas histone chloride is readily precipitated by acetone but only poorly by ethanol.

The histone sulphate was finally washed three times each with ethanol and ether, and air dried.

II. Isolation of Basic Proteins from Micro-organisms

The basic proteins of micro-organisms were isolated by acid extraction, followed by purification involving dialysis and separation on columns of carboxymethyl-cellulose (CM-cellulose). The results leading to these purification steps are summarised and discussed in pp. 57 - 62.

The extraction procedure described below was developed for whole bacteria, and modifications introduced for other organisms are given in the experimental sections. The dialysis and CM-cellulose separation, also developed for purifying material of bacterial origin, were subsequently used routinely without modification for the purification of basic proteins extracted from various sources.

The organisms to be studied were stored in the deep freeze until required. After thawing, the material was suspended in 6 volumes of cold 4% acetic acid and centrifuged for 15 min. at 10,000 x g in an M.S.E. "17,000" refrigerated centrifuge. The supernatant was discarded, and the sedimented material resuspended in 6 volumes of

1% acetic acid. The organisms were washed at least twice with 1% acetic acid by repeating the centrifugation and resuspension processes. These washes remove any remaining culture medium, and ensure a fairly acid pH. The material was then defatted with three washes each of ethanol and ether, and air dried.

i. Extraction Procedure (Bacteria)

The dry organisms were weighed out and transferred to a porcelain mortar. For each g. of material, 2 g. fine glass powder and 3 ml. 0.1$\bar{N}$ sulphuric acid were added. This mixture was ground vigorously with a pestle in the cold room for 5 min. and then left to stand for 3 hr. During this time the grinding process was repeated about five times, so ensuring adequate disintegration of the organisms. Finally, after adding a further 3 ml. 0.1$\bar{N}$ sulphuric acid per g. of organisms, the extraction was allowed to continue for a further 3 hr. The glass powder and cell debris were then removed by centrifugation at 27,000 x g for 30 min. in the M.S.E. "17,000" refrigerated centrifuge.

This extraction procedure was repeated at least twice, using another 3 ml. 0.1$\bar{N}$ sulphuric acid per g. of organisms on each occasion, and the supernatants were combined.

ii. Dialysis

The combined extracts were transferred to dialysis tubing (Visking Cellulose Tubing, $\frac{18}{32}$" diameter) and dialysed against large volumes of 0.1$\bar{N}$ sulphuric acid in order to remove substances of low molecular weight. The dialysed solution was normally added to 8 volumes of ethanol to precipitate the protein sulphates, which were collected by centrifugation, and air dried after three washes each of ethanol and ether.

In the later preparations the precipitation stage was omitted. The dialysed solution was prepared for applying to CM-cellulose by continuing the dialysis against large volumes of distilled water (6 hr.) and then against the pH 7.0 bicarbonate buffer (described below) for 12 hr. Any material precipitated was removed by centrifugation.

iii. Separation of Basic Proteins on Carboxymethyl-cellulose

The weak cation exchanger, CM-cellulose, which was first described by Peterson and Sober (1956), is well suited to the purification of

basic proteins. On applying a solution, previously adjusted to pH 7, to a column of CM-cellulose, the non-basic components run through while the basic components are retained. The basic material can then be eluted with a small volume of acid, thus both concentrating and purifying the material in a single step.

A suspension of CM-cellulose (Whatman Powder CM 70) in water was poured into a 1.0 cm. diameter glass column fitted with a tap. The CM-cellulose was packed to a height of 3 cm. with a glass plunger, using only light pressure. The column was then washed successively with 0.1$\bar{N}$ hydrochloric acid, water, and bicarbonate buffer pH 7.0.

Bicarbonate buffer was chosen as the anion is not multi-charged, and it was therefore considered less likely to cause protein interactions. Similarly hydrochloric acid was used for eluting the bound proteins. The bicarbonate buffer was prepared by adding small pieces of solid carbon dioxide to a solution of 0.01$\bar{M}$ sodium bicarbonate until the pH had fallen to 7.0 (as measured on a pH meter). This buffer was always kept in tightly stoppered bottles, and the pH checked regularly.

The dry material, obtained after the dialysis, was dissolved in bicarbonate buffer at a concentration of approximately 50 mg. per ml. The pH was then adjusted to 7.0 (Johnson's narrow range indicator paper) by the addition of sodium hydroxide, and any insoluble material removed by centrifugation. This pH adjustment produces some sodium sulphate, and hence for the later preparations adjustment by dialysis was preferred.

The pH 7.0 protein solution was then run on to the CM-cellulose column at approximately 2 ml. per min. The column was washed with 100 ml. of the bicarbonate buffer, followed by 100 ml. distilled water. The basic protein bound to the column was eluted with 0.1$\bar{N}$ hydrochloric acid, 2 ml. fractions of the eluate being collected. The optical densities of the fractions were read at 276 mμ. against water in a Unicam SP 500 spectrophotometer (0.5 cm. light path). Fractions with optical densities above 0.02 were pooled and then dialysed against 0.1$\bar{N}$ sulphuric acid. This dialysis was necessary to remove the salt which was simultaneously eluted from the column, and also served to exchange the acids, so enabling the protein sulphates to be precipitated. The protein

was precipitated by adding 8 volumes of ethanol to the dialysed solution, or, when quantitative recovery was desired, by dialysis against ethanol. The protein sulphate was finally washed in ethanol and ether, and air dried.

iv. Determination of Dry Weights and Yields

Unless otherwise stated, all dry weights of micro-organisms and their sub-fractions, and yields of basic proteins, were determined gravimetrically by weighing the "air dried" material after successive washes with ethanol and ether.

III. Method of Starch Gel Electrophoresis

Zone electrophoresis in starch gel supporting medium was based on the method of Smithies (1955). Acid hydrolysed ("Smithies'") starch was prepared from potato starch (B.D.H.) as described by this author.

Acetate buffer, 0.028M and pH 4.75 was used throughout. This was prepared as required by diluting a 0.40M stock solution (containing 27 g. hydrated sodium acetate and 11.75 ml. glacial acetic acid per litre), kept sterile by the addition of a few drops of chloroform.

According to the adaptation of Neelin and Neelin (1960) for histones, 4M urea was incorporated into the gels, and approximately 16% starch was used. For each batch of starch, the most suitable concentration for preparing the gels was determined by trial experiments.

On account of the small quantities of protein often obtained during this investigation, a micro-method of starch gel electrophoresis was developed. Shallow gels were rolled out 1 mm. thick on glass plates, thus enabling electrophoresis to be performed on less than 0.2 mg. of protein.

Figure 2 a.

Arrangement of glass plate and microscope slides for casting starch gel.

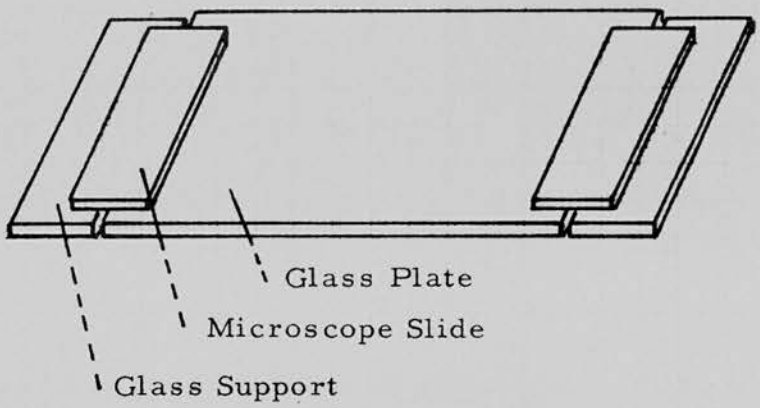


Figure 2 b.

Slot Cutter

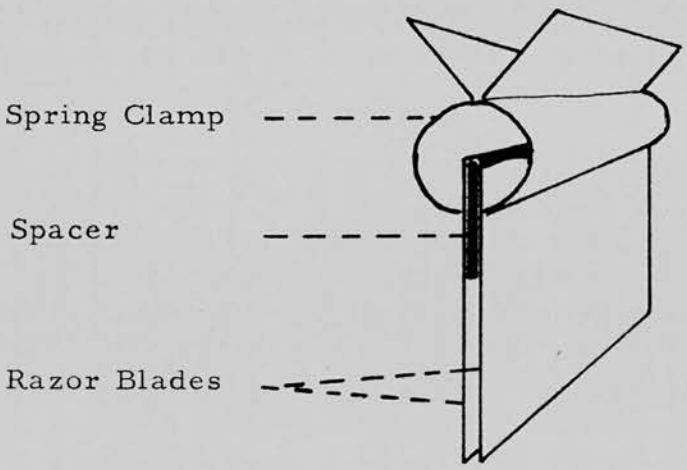
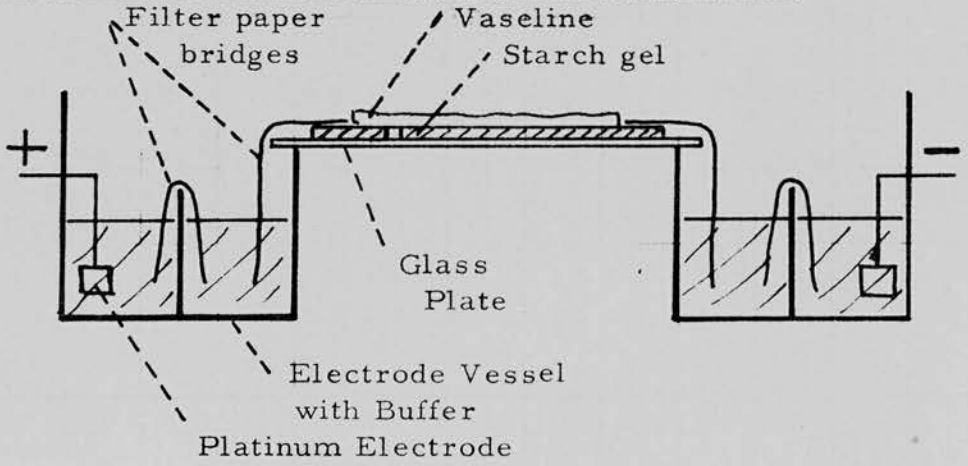


Figure 2 c.

Arrangement of apparatus during electrophoresis



Two microscope slides (7.5 cm. x 2.5 cm. x 1mm) were attached by means of "sellotape" to a glass plate (12 cm. x 9 cm. x 1 mm.) as shown in Fig. 2a. The overlapping edges of the slides were supported underneath by two further pieces of 1 mm. thick glass, and the upper surface of the glass plate was smeared very thinly with liquid paraffin.

The gel was prepared by adding the buffer (40 ml.) to the starch and urea in a 150 ml. conical flask, and heating the mixture in a boiling water bath with constant stirring until the temperature had been maintained within the range 95 - 98°C for approximately 2 min. Air bubbles were removed from the viscous solution by evacuating the flask at a water pump for 5 to 10 sec. and swirling vigorously. The hot solution was then poured without delay on to the glass plate between the two slides, and covered with a thin sheet of polythene previously coated lightly with liquid paraffin. The still liquid starch solution was then rolled out flat to the thickness of the slides. This was achieved by running a heavy roller up and down along the slides, and so forcing the excess gel out from under the polythene at the edges of the glass plate. Another glass plate was then placed over the polythene sheet, and a weight added to that.

The pH of any gel prepared in this way was found to be between 5.5 and 6.0. The pH measurement was made on a small portion of the gel cooled separately in a beaker. Half a volume of distilled water was added and the gel broken up by stirring with a glass rod. After standing for at least 1 hr. the pH of this mixture was read on a pH meter. Values obtained in this way were in good agreement with those indicated by inserting a piece of narrow range indicator paper into the gel.

After the gel had been allowed to set for at least 1 hr. the weight, the upper glass plate, and the polythene sheet were carefully removed. The gel was trimmed to the width of the glass plate by removing the excess gel at the edges with a scalpel.

A small instrument (Fig. 2 b) was devised to aid in making the sample slots. This consisted of two 1.2 cm. widths of razor blade, separated by a 1 mm. thick spacer, and clamped together with a small "bulldog" clip. This instrument was used to produce two parallel cuts in the gel. The small intervening piece of gel was then readily removed with a 1 mm. wide strip of celluloid, leaving a neat sample slot (1.2 cm. x 1 mm. x 1 mm.).

Normally four such slots, 0.8 cm. apart, were formed in a line across the gel, at a distance of approximately 2.5 cm. from one of the slides.

The protein samples were prepared for application in micro test-tubes, made by sealing one end of 2 cm. lengths of 3 mm. (internal diameter) glass tubing. The protein (approximately 0.2 mg.) was dissolved in about 10 µl. acetate buffer. Urea was added to make the solution approximately 4 M, and then fine starch grains were mixed in until a thin paste was obtained. (The fine starch grains had previously been prepared by fractionating B.D.H. potato starch. The fraction not settling out from an aqueous suspension in 5 min. was collected and dried, the heavier grains being discarded).

The protein samples were introduced into the slots by means of fine capillary pipettes. Care was taken to fill the slots until the level of the sample was just flush with the upper surface of the gel.

Approximately equal quantities of white "vaseline", liquid paraffin, and petroleum ether were mixed together and allowed to stand in hot water. A warm syringe was then used to spread

this low melting point grease over the gel. Only two 1 cm. wide strips of the gel next to the glass slides were left uncovered, thus allowing electrical contact with the paper bridges during electrophoresis.

After removing the two glass microscope slides, the gel was set up for electrophoresis as shown in Fig. 2 c. Platinum electrodes were used with reservoirs of 600 ml. capacity, and the buffer was renewed after each run. The bridges were formed by six layers of Whatman No.1 filter paper soaked in the acetate buffer.

Electrophoresis was carried out at a potential gradient of 1.5 v/cm. for 16 hr. at room temperature. These conditions gave a current of 1.5 ma. Because of the thinness of the gel and the low potential gradient, there was little increase in temperature during electrophoresis and cooling was therefore unnecessary.

At the completion of the run, the gel, still resting on the glass plate, was submerged in four washes of warm petroleum ether in order to remove the "vaseline" coating. It was then transferred to a saturated solution of Amido Black 10 B (Gurr, Ltd. London) in methanol:glacial acetic acid:water

(5:1:5 by volume) for 5 min. During this time the gel could normally be coaxed free from the glass plate, thus allowing staining on both sides. Excess stain was removed by several washes of the same solvent over a period of some 12 hr.

The gels could be preserved for a period of several weeks, with little fading of the stained bands, by immersing in a mixture of the above solvent and glycerol (1:1 by volume).

Permanent records of gels were obtained by photographing with a standard photo-copying camera, using ortho-plates (Ilford N40).

IV. Method for the Analysis of Arginine, Lysine, Glutamic Acid, and Aspartic Acid Residues in Proteins

i. Hydrolysis of Proteins

Protein samples were dried to constant weight by keeping in a vacuum desiccator over P_2O_5 for a period of several days.

When sufficient protein was available, approximately 5 mg. of the samples were weighed out accurately and transferred to 10 ml. flasks. After adding 2 ml. of 6N HCl to each flask, reflux condensers were attached, and the solutions refluxed gently for 24 hr.

HCl and H_2O were removed after hydrolysis by heating under vacuum in a water bath, and finally by placing the flasks in a desiccator over P_2O_5 and NaOH. To ensure complete removal of HCl, the hydrolysates were redissolved in approximately 1 ml. of water, and taken down to dryness as before. The hydrolysates were then dissolved carefully in exactly 1.0 ml. water, and these solutions were transferred to stoppered specimen tubes and stored in the deep freeze.

When only small quantities of protein were available for analysis, it was found more convenient to carry out the hydrolysis in sealed tubes. 7 cm. lengths of 4 mm. (internal diameter) tubing were sealed at one end. Approximately 1 mg. of the samples were weighed out and transferred to these tubes. 0.4 ml. of 6$\bar{N}$ HCl was added to each tube, which was then sealed and placed upright in an oven at 105°C for 24 hr. After hydrolysis the upper sealed ends of the tubes were cut off, and the HCl and H_2O removed in the desiccator over P_2O_5 and NaOH. The hydrolysates were redissolved in a small volume of water and the drying procedure repeated. The dry hydrolysates were finally dissolved in 0.25 ml. water, and the tubes stoppered before storing in the deep freeze.

ii. The Separation and Estimation of Amino Acids Using Low Voltage Paper Electrophoresis.

A method for the determination of ornithine and citrulline in tissue extracts has been developed by Bronk and Fisher (1956). The amino acids were separated by high voltage paper electrophoresis, and the ninhydrin-copper method of Harris and Warren (1954) was used for their estimation.

Evered (1959) has demonstrated that various amino acids can be readily separated by low voltage paper electrophoresis. In particular arginine and lysine are separated at pH 11.5, and glutamic and aspartic acids at pH 4.0.

As the low voltage technique has the advantage that it does not require such expensive or elaborate apparatus, it was decided to estimate arginine, lysine, glutamic acid and aspartic acid in protein hydrolysates by combining the low voltage separations with the ninhydrin-copper method.

Two standard amino acid mixtures were used during the analyses. These were prepared in distilled water from the solids (Roche Products Ltd., Welwyn Garden City) which had been dried *in vacuo* over P_2O_5.

Solution *a* contained 5 μ mole arginine and 5 μ mole lysine hydrochloride per ml.

Solution *b* contained 5 μ mole glutamic acid and 5 μ mole aspartic acid per ml.

These standard solutions were stored in the deep freeze until required.

Method of Low Voltage Paper Electrophoresis.

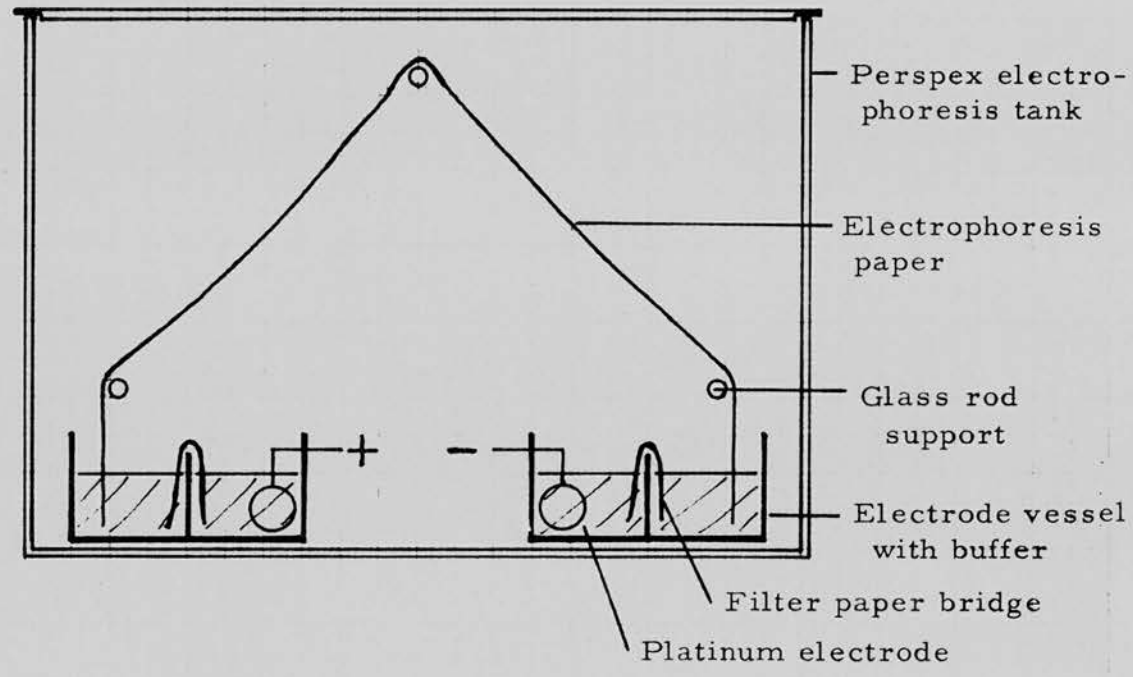
A large clean glass plate was used as a working surface for the preparation of the electrophoresis papers.

A large sheet of Whatman No. 1 filter paper (chromatography grade) was cut into a strip 54 cm. long and 23 cm. wide. A light pencil line was drawn across the centre of the strip, and seven positions for spotting the amino acid mixtures were marked and numbered at equal distances across this line.

Four samples of protein hydrolysates and three of a standard amino acid mixture were put onto each paper. 10 µl. of the hydrolysates, and 5 to 30 µl. of the standard mixture (containing 0.025 to 0.150 µ mole of each amino acid) were run onto the paper from capillary pipettes. Care was taken to keep the spots as small as possible by adding small portions of the solutions at a time, and drying the spots with a hair drier between additions. During this procedure the paper was kept clear of the working surface with suitably placed glass rods.

Figure 3

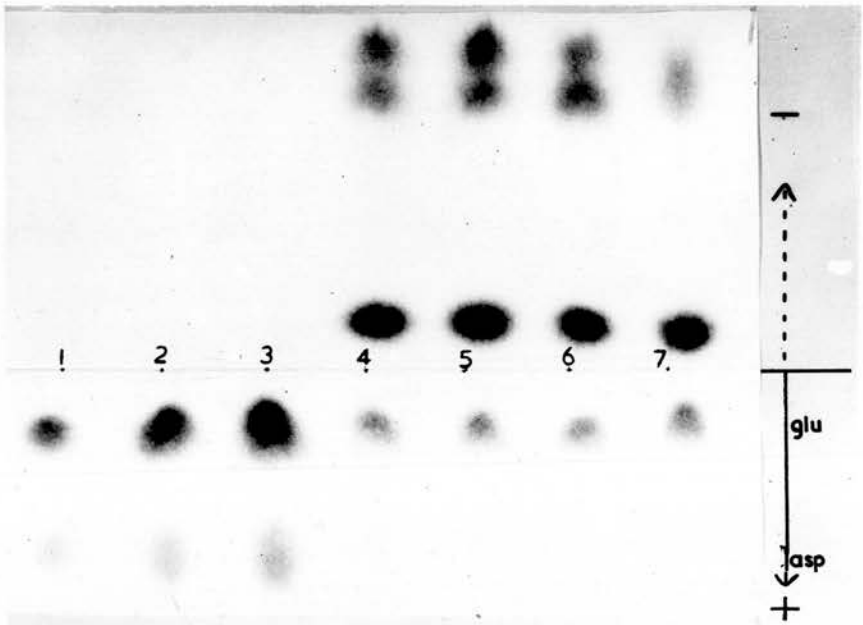
Low Voltage Paper Electrophoresis Apparatus



The electrophoresis apparatus was set up as shown in Fig. 3. Each compartment of the electrode vessels contained 300 ml. of the appropriate buffer, and the two compartments of each vessel were bridged with 23 cm. wide filter paper wicks. The centre of the electrophoresis paper was supported by the upper glass rod, and its extremities were passed over the lower rods and dipped into the buffer in the outer compartments of the electrode vessels. Buffer solution was then run from a pipette over the sloping surface of the electrophoresis paper to within 2 cm. on either side of the line of sample spots situated at its highest point. If necessary further small additions of buffer were made just below the samples to ensure that the buffer fronts approached the samples from opposite sides at the same instant. The lid was then placed on the apparatus and the current switched on.

In order to separate glutamic and aspartic acids, 0.05M potassium hydrogen phthalate pH 4.0 was used as the buffer solution, and the electrophoresis was carried out at a potential gradient of 8 v/cm. for a period of 4 hr. A photograph

Figure 4.



ELECTROPHORETIC SEPARATION OF GLUTAMIC AND ASPARTIC ACIDS IN PROTEIN HYDROLYSATES.
BUFFER — 0·05M POT. HYD. PHTHALATE pH 4·0.
4 HOURS AT 8·0 VOLTS/CM.

1–3. GLU. AND ASP. STANDARDS.
4. B. MEGATERIUM BASIC PROTEIN.
5. UNFRACTIONATED CALF THYMUS HISTONE.
6. β FRACTION CALF THYMUS HISTONE.
7. INSULIN.

Figure 5.

ELECTROPHORETIC SEPARATION OF ARGININE AND LYSINE IN PROTEIN HYDROLYSATES.
BUFFER — 0·05M SODIUM CARB/BICARB. pH 10·5.
6 HOURS AT 7·5 VOLTS/CM.
1.—3. ARG. AND LYS. STANDARDS.
4. B. MEGATERIUM BASIC PROTEIN.
5. UNFRACTIONATED CALF THYMUS HISTONE.
6. β FRACTION — CALF THYMUS HISTONE.
7. INSULIN.

of this separation, in which the amino acids spots were developed by the ninhydrin-copper method, is shown in Fig. 4.

It was found that arginine and lysine could be most effectively separated at pH 10.5, rather than at pH 11.5 as suggested by Evered (1959). 0.05M carbonate buffer pH 10.5 was used, and the electrophoresis was carried out at a potential gradient of 7.5 v/cm. for 6 hr. The buffer was prepared by adding 300 ml. of 0.05M $NaHCO_3$ to 1000 ml. of 0.05M Na_2CO_3. The separation achieved is shown in Fig. 5.

The potential gradients used were the maximum which could be obtained from the available D.C. power supply. Although these potentials are considerably higher than the 5 v/cm. used by Evered, no excessive heating of the papers was detected.

Following completion of the electrophoresis, one end of the paper was attached with clips to a glass rod in order to facilitate its removal from the apparatus. The paper was then hung in a chromatogram drying oven at about 70°C for 30 min. It was then ready for putting through the ninhydrin-copper method.

Ninhydrin-Copper Method

Two solutions are required:

1. Ninhydrin Reagent -

   Either 0.5 g. ninhydrin; 95 ml. acetone; 5 ml. H_2O (used after electrophoresis at pH 4.0)

   or 0.5 g. ninhydrin; 90 ml. acetone; 5 ml. acetic acid; 5 ml. H_2O (used after electrophoresis at pH 10.5)

2. Cupric Nitrate Reagent -

   2 ml. saturated cupric nitrate, 0.4 ml. 10% v/v nitric acid, made up to 100 ml. with ethanol

For spraying electrophoresis papers a 50 ml. capacity surgical throat spray attached to the compressed air line was used. The dry paper to be analysed was hung in a fume cupboard, and kept vertical by heavy clips attached to the lower end. 50 ml. of the ninhydrin reagent was sprayed evenly over the surface of the paper. After the solvent had evaporated the fan was stopped, the fume cupboard closed in order to protect the paper from contamination, and the ninhydrin colours allowed to develop overnight. The paper was also protected from direct daylight at this stage.

After approximately 16 hr. the paper was sprayed evenly with 50 ml. of the cupric nitrate reagent, and allowed to stand for a further 30 min. at room temperature. The cupric nitrate causes the typical ninhydrin colours to change to a very stable reddish-pink colour. The background colour due to the reagent is a light greenish-blue.

The coloured spots, corresponding to the particular amino acids being separated, were then cut out and placed in dry 10 ml. tapered centrifuge tubes. Each spot was cut out on approximately the same area of paper, and two similar areas containing no ninhydrin reacting material were cut out as reagent blanks, in order to allow for the background colour.

5 ml. methanol was measured into each centrifuge tube. The tubes were then stoppered and kept at room temperature for 2 hr. to allow extraction of the coloured spots. During this period the tubes were gently shaken two or three times, and at the end of the 2 hr. period the colours had been completely extracted.

ABSORPTION SPECTRA OF NINHYDRIN-COPPER COMPLEXES OF AMINO ACIDS. EACH SPOT ELUTED FROM ELECTROPHORESIS PAPER INTO 5 ml. METHANOL. 0.15 μ MOLE OF EACH AMINO ACID. SOLUTIONS READ AGAINST METHANOL.

Figure 6 a. Glutamic Acid and Aspartic Acid

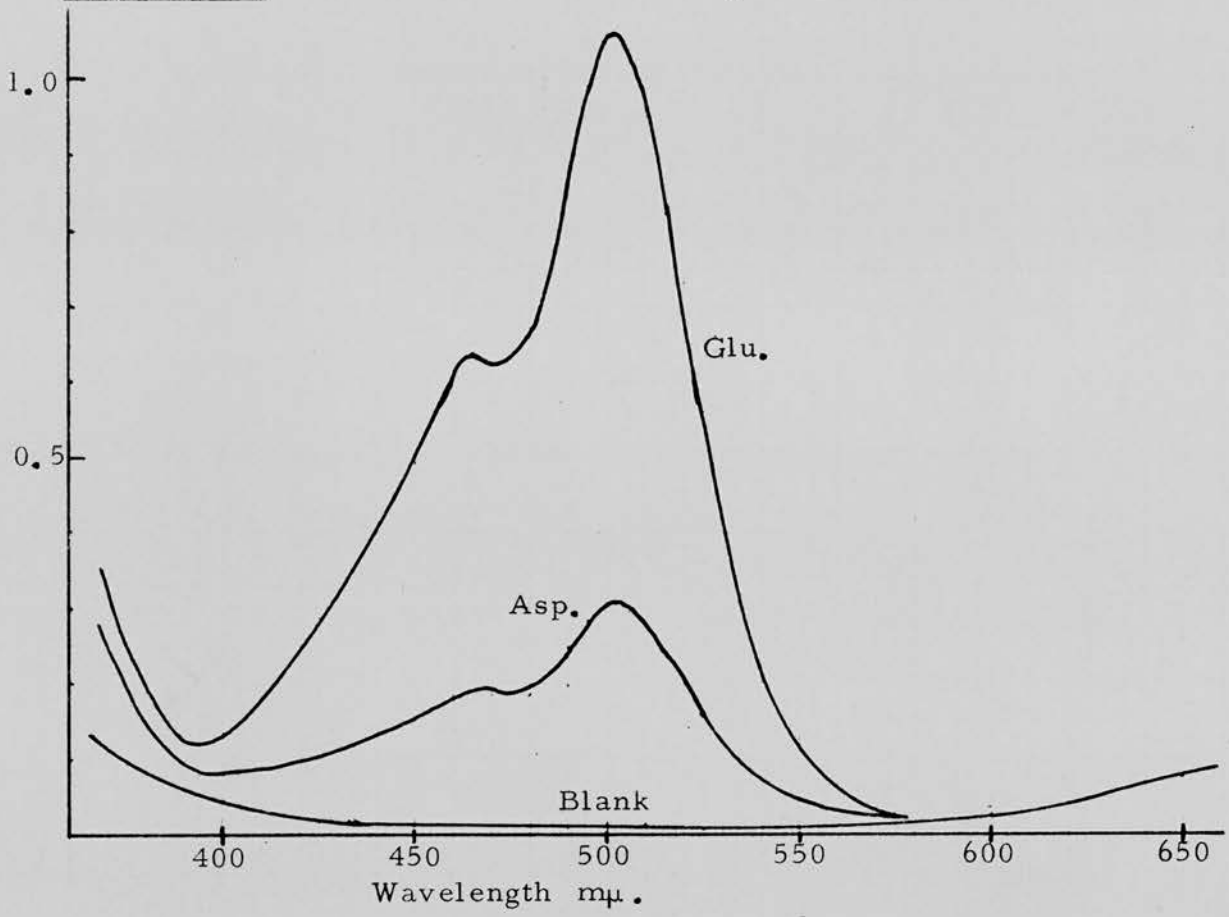
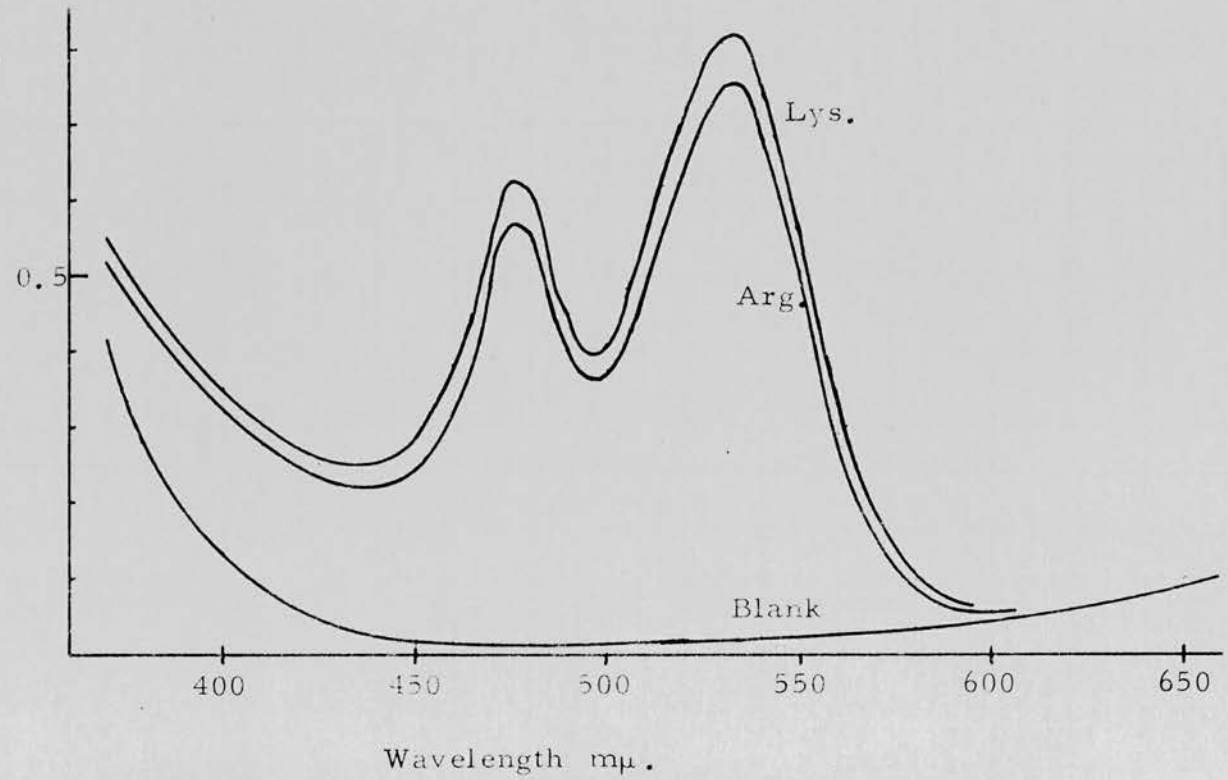


Figure 6 b. Lysine and Arginine



The bulk of the filter paper was then wedged into the narrow foot of the tubes with a glass rod, and the remaining suspended paper fibers were sedimented by centrifuging at 3,000 x g for 2 min. The optical densities of the clear solutions were then read against methanol in a Unicam SP500 spectrophotometer.

It was found that the colours obtained at the two different pH's were rather different - the pH 10.5 paper giving a slightly deeper red. The absorption spectra of the colours obtained with glutamic and aspartic acids after the pH 4.0 separation are shown in Fig. 6 a, and with arginine and lysine after separation at pH 10.5 in Fig. 6 b. After the electrophoresis at pH 4.0, the ninhydrin-copper complexes of the amino acids absorb maximally at 504 mμ. - in fair agreement with Bronk and Fisher (1956) who measured the optical densities at 510 mμ. after separation at pH 3.2. However, after electrophoresis at pH 10.5 the maximum absorption is shifted to 535 mμ. If the pH 10.5 paper was neutralised before staining the maximum absorption returned to 504 mμ. but the colours became unstable and faded rapidly.

Arginine and lysine have therefore been estimated by measuring the optical densities of the ninhydrin-copper complexes at 535 mμ. while the glutamic and aspartic acid optical densities were read at 504 mμ.

The absorption spectra of the background colours ("blanks"), after both separations, show very low absorption at the wavelengths where the amino acid complexes absorb. Any slight variations in the background colour will therefore have little effect on the accuracy of the estimation procedure. Two background colours have been read during each estimation and the average "blank" value subtracted from the readings of the amino acid - ninhydrin-copper colours.

It was found that the colours obtained with standard amino acid solutions varied in intensity between different runs, although for each run a linear calibration curve was obtained. It was therefore essential to put standards on every paper, and to plot a calibration graph for every run.

Calibration curves for the determination of glutamic and aspartic acids (Fig. 7 a) were linear

Figure 7 a.

Calibration Curves for the Estimation of Glutamic and Aspartic Acids

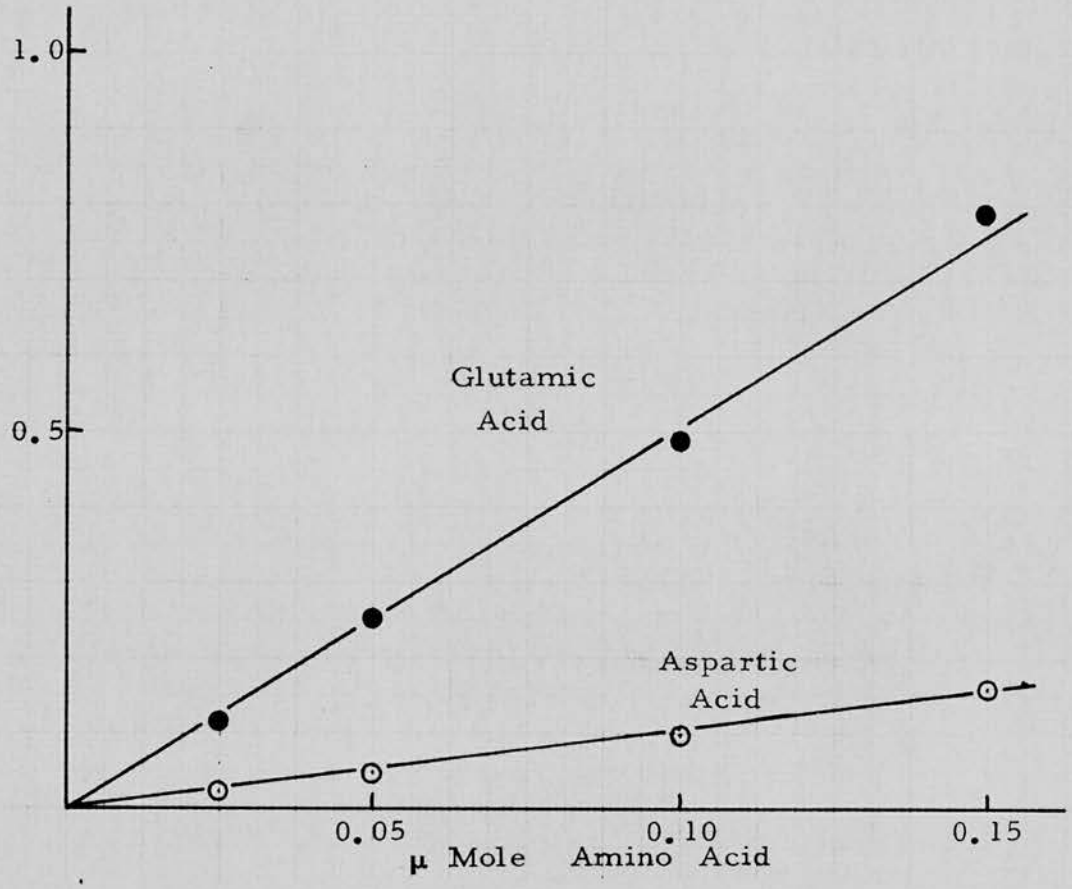
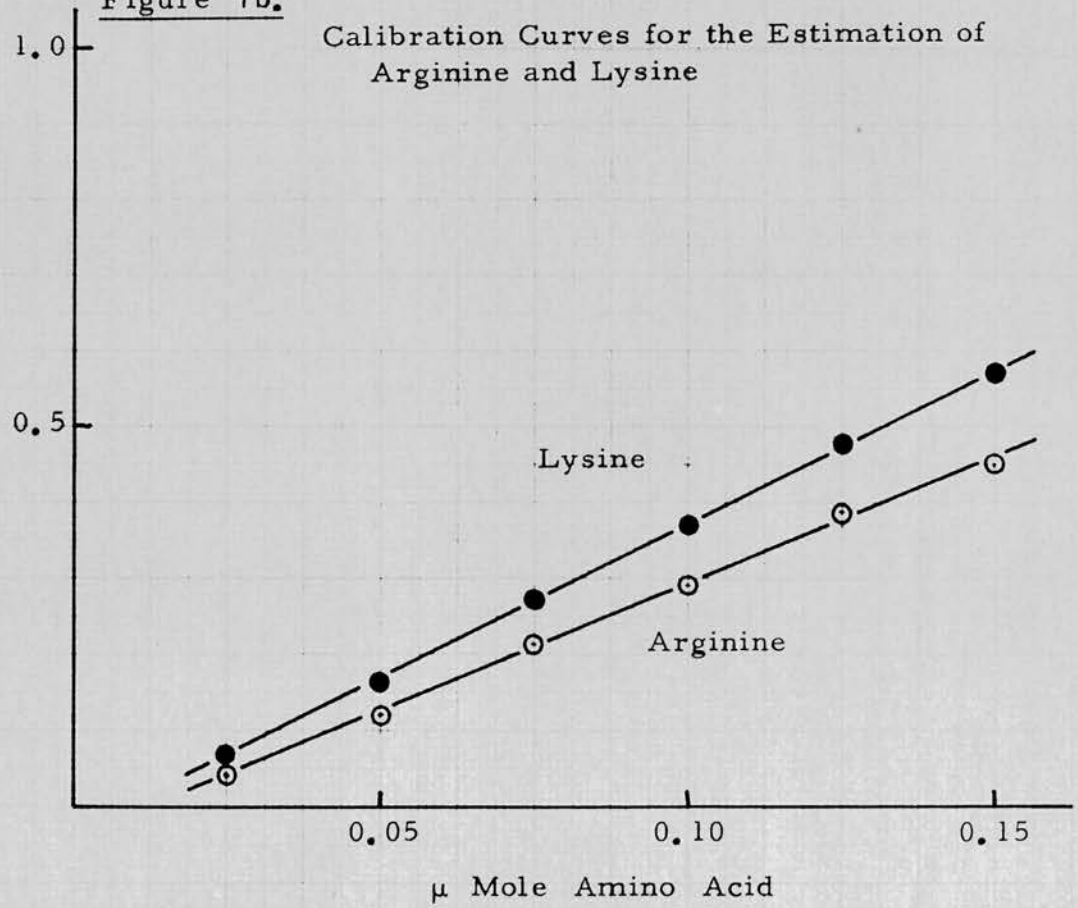


Figure 7b.

Calibration Curves for the Estimation of Arginine and Lysine



over the range 0 to 0.15 μ mole per spot. Quantities of these amino acids determined were kept within the range 0.025 to 0.150 μ mole.

The calibration curves for the determination of arginine and lysine (Fig. 7 b) were linear over the range 0.025 to 0.150 μ mole per spot. However these lines if extrapolated usually failed to pass through the origin, indicating that below 0.025 μ mole the calibration is non linear. It was therefore essential that the quantity of arginine or lysine to be estimated was kept above 0.025 μ mole.

All results for amino acid analyses of proteins were calculated as g. amino acid residue in 100 g. protein.

iii. Trial Analyses of Amino Acid Residues in Proteins

In order to test the procedure for the analysis of arginine, lysine, glutamic acid and aspartic acid residues in basic proteins, unfractionated calf thymus histone and a sample of calf thymus β histone fraction prepared by Dr. H.J. Cruft were analysed. The results for

TABLE 2

Analysis of arginine, lysine, glutamic acid and aspartic acid residues in proteins

All results expressed as g. amino acid residue in 100 g. protein.
Average values of three analyses of each residue are given.

Amino acid residue	Unfractionated Histone		β-Histone Fraction		Insulin	
		A		B		C
Arginine	10.7 (10.5,10.9,10.6)	10.3	15.0 (13.6, 16.1, 15.2)	16.1	2.0 (1.2, 2.2, 2.5)	2.7
Lysine	15.6 (15.3, 16.1, 15.4)	15.5	10.1 (9.7, 10.4, 10.1)	9.9	2.1 (1.5, 2.3, 2.6)	2.2
Glutamic acid	7.6 (7.5, 7.7, 7.5)	8.5	10.3 (9.7, 11.2, 10.0)	10.7	13.5 (13.4, 13.7, 13.4)	15.7
Aspartic acid	6.0 (7.1, 6.7, 4.1)	4.4	4.8 (5.3, 4.6, 4.6)	5.7	5.0 (4.8, 5.5, 4.6)	6.0

A. Data of Crampton, Moore and Stein (1955) converted to g. amino acid residue/100 g. protein
B. Data of Cruft, Mauritzen and Stedman (1958)
C. Values for pure beef insulin determined from its structure

the former were compared with the data of Crampton, Moore and Stein (1955) converted to g. amino acid residue per 100 g. protein. The values obtained for the β histone were compared to those published by Cruft, Mauritzen and Stedman (1958) for this protein. Recrystallized insulin (Boots Pure Drug Co. Ltd.) was also analysed, and the results compared to the theoretical values for pure beef insulin as determined from its structure. A single sample of each protein was hydrolysed, and three estimations of each of the four amino acids were performed. These results, together with the data for comparison, are summarised in Table 2.

The results for the unfractionated histone and the β histone fraction compare favourably with the published data. The method therefore appears suitable for determining whether proteins isolated from micro-organisms are similar to histones in their content of these four amino acids. Although there is some degree of scatter in the values of the three analyses, this is not large enough to invalidate any conclusions about the histone-like nature of the proteins studied, particularly as no precise analysis can be quoted as characteristic of a histone (p. 3).

The insulin results are in fair agreement with the theoretical values, although they do appear to have a tendancy to be low. It was considered that this might be because the sample analysed was not 100% pure. Incomplete removal of water, by the treatment *in vacuo* over P_2O_5 is certainly a possibility.

V. Examination for the Presence of Cystine in Protein Hydrolysates

In order to determine if proteins contained cysteine or cystine, low voltage paper electrophoresis, as described above, was carried out using 0.05M veronal buffer pH 8.6. At this pH cystine is separated from amino acid mixtures (Evered, 1959). The buffer was prepared by dissolving 10.3 g. Na diethylbarbiturate and 1.84 g. diethylbarbituric acid in water, and making up to 1 litre. Electrophoresis papers were run at 10 v/cm. for 4 hr. and the amino acid spots detected by the ninhydrin-copper method already described. Standard spots were run simultaneously, and the stained papers were examined visually. It was found that amounts of cystine down to 0.001 μ mole per spot could be detected.

VI. Ultra-violet Absorption Curves of Proteins

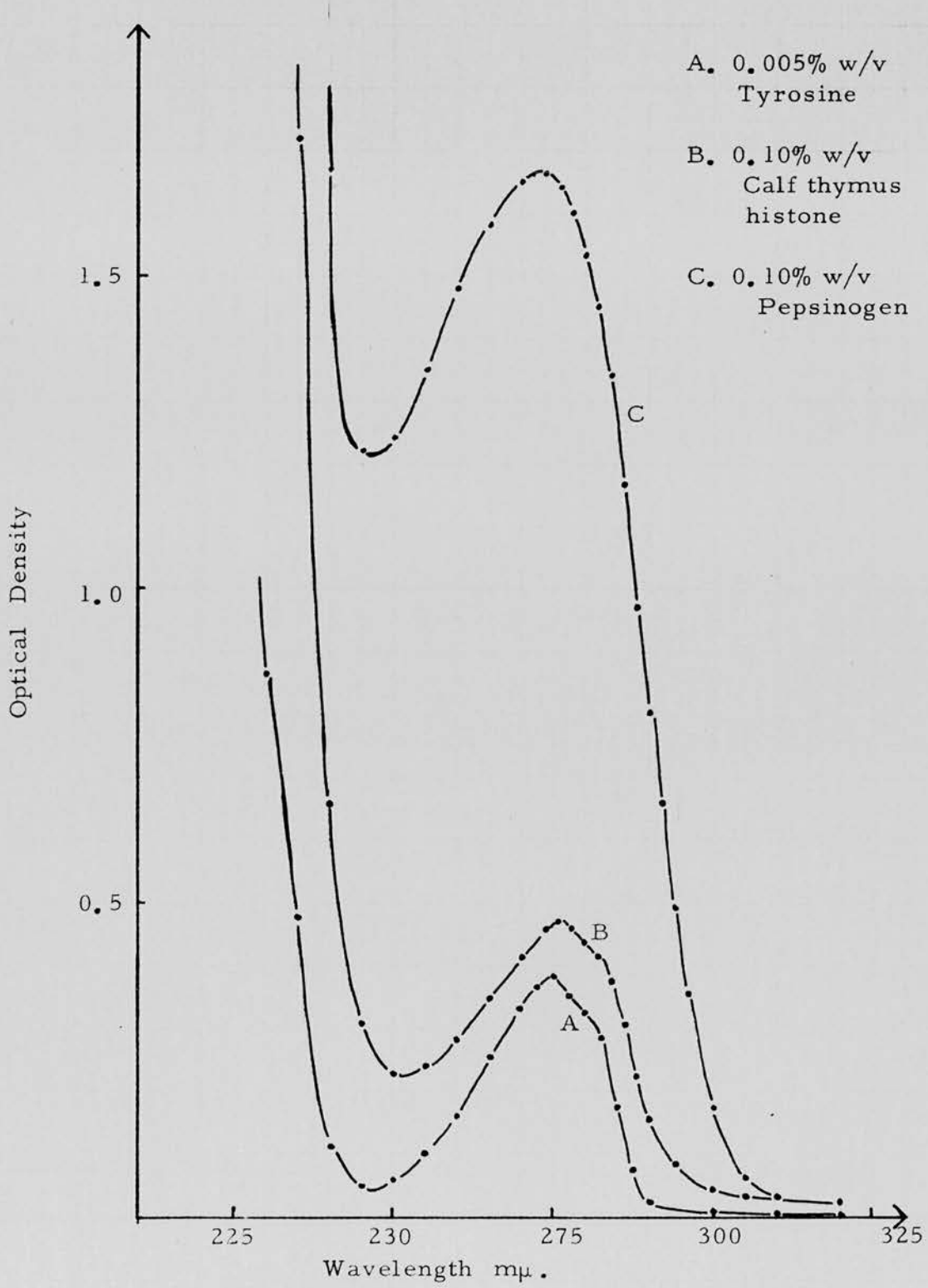
All optical density measurements were taken on a Unicam SP500 spectrophotometer, using either 0.5 cm. or 1 cm. silica cells.

An approximately 0.10% w/v solution of the protein was prepared by weighing out the protein (dried *in vacuo* over P_2O_5) and dissolving in the solvent, which was usually 0.1N H_2SO_4. Optical densities were read against the solvent over the wavelength range 230 to 320 mμ.

Occasionally it was more convenient to determine the ultra-violet absorption curves at the last stage in the purification of proteins. In this case the volume of the acid solution was carefully measured and after the readings had been taken, the protein was precipitated by dialysis against 100% ethanol. The protein was carefully removed from the dialysis sac, washed with ether, dried in air and finally *in vacuo* over P_2O_5, and then weighed.

Figure 8.

Ultra-violet Absorption Curves
Unicam SP 500 - 1.0 cm light path.



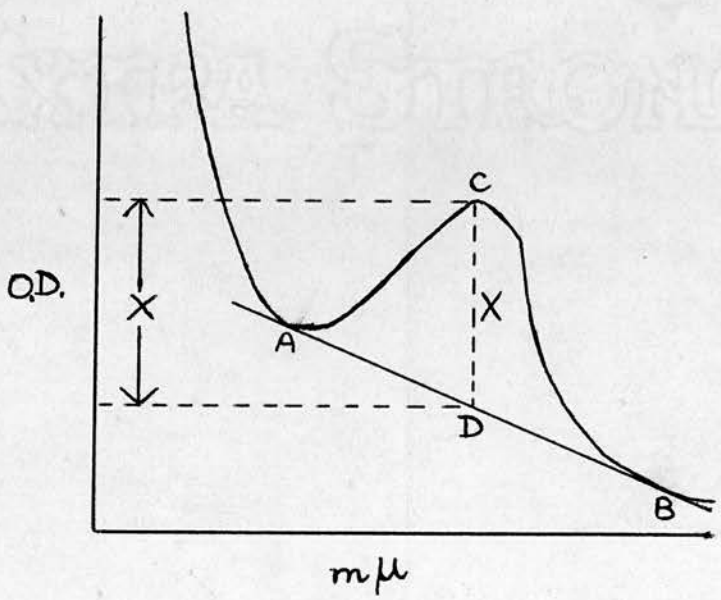
The ultra-violet absorption curves of proteins have been studied in order to determine if many tryptophan residues are present, and where possible to make an estimate of the tyrosine content.

Proteins of the histone type, which contain tyrosine but no tryptophan, exhibit absorption curves which are very similar to that of free tyrosine, and show a maximum absorption at 276 mμ. (Fig. 8). The main deviation from the tyrosine curve is a greater apparent absorption which increases progressively as the wavelength shortens. Cruft, Mauritzen and Stedman (1957) consider that this effect is due to light scattering (Tyndall phenomenon) which becomes greater at shorter wavelengths.

The extinction of a tryptophan solution is more than three times that of a tyrosine solution of the same w/v concentration (Cruft, 1953). Hence proteins which contain even a small proportion of tryptophan exhibit a much stronger absorption peak, which does not have the characteristic shape of the tyrosine curve, and in which the maximum is often slightly shifted from 276 mμ.

This is demonstrated in Fig. 8 where the absorption spectrum of pepsinogen, containing 2.8% w/w tryptophan residues and 6.8% w/w tyrosine residues (Arnon and Perlmann, 1963), is compared to those of calf thymus histone and tyrosine.

Provided the u.v. absorption curve of a protein is similar to that of tyrosine and gives no indication of the presence of tryptophan, then it can be used to estimate a minimum tyrosine content for the protein as described by Cruft (1953).



A straight line is drawn as a tangent to the two low proportions of the curve, A and B. Then the vertical line CD is dropped from the maximum at 276 mμ. on to this line. The distance X (CD) is read from the optical density scale and used to represent the amount of the absorption due to tyrosine.

Cruft (1953) showed that the value of X for the absorption curve of a 0.030% w/v solution of tyrosine in 0.1$\bar{N}$ H_2SO_4 was 1.07 optical density scale (0.5 cm. light path). Using this figure, the value of X obtained for a protein solution is converted to a figure for the % w/v tyrosine in the solution, and by knowing the protein concentration as a % w/v, the tyrosine figure can then be expressed as a weight percentage of the protein.

VII. Estimation of Nucleic Acids in Microbiological Materials

DNA and RNA present in microbiological materials have been estimated by analysing perchloric acid extracts. These were prepared by extracting the weighed material (previously defatted and dried) three times with 0.5M perchloric acid at 70°C for 15 min. The insoluble material was removed after each extraction by centrifugation, and the combined supernatants were made up to a suitable volume with more perchloric acid for analysis. The conditions for extraction were chosen because Burton (1956) has shown that 96% of the DNA is extracted from Escherichia coli by two such extractions, and because destruction of DNA is minimal when perchloric acid at 70°C is the extractant (Hutchison and Munro, 1961). RNA is readily extracted from biological material by hot perchloric acid, and the ribose is not subject to any degradation (Hutchison and Munro, 1961).

DNA was determined in the extracts by the diphenylamine reaction, and RNA by the orcinol reaction of Dische and Schwartz, both as described by Dische (1955).

Figure 9 a.

Calibration Curve for the Diphenylamine Method of DNA Estimation

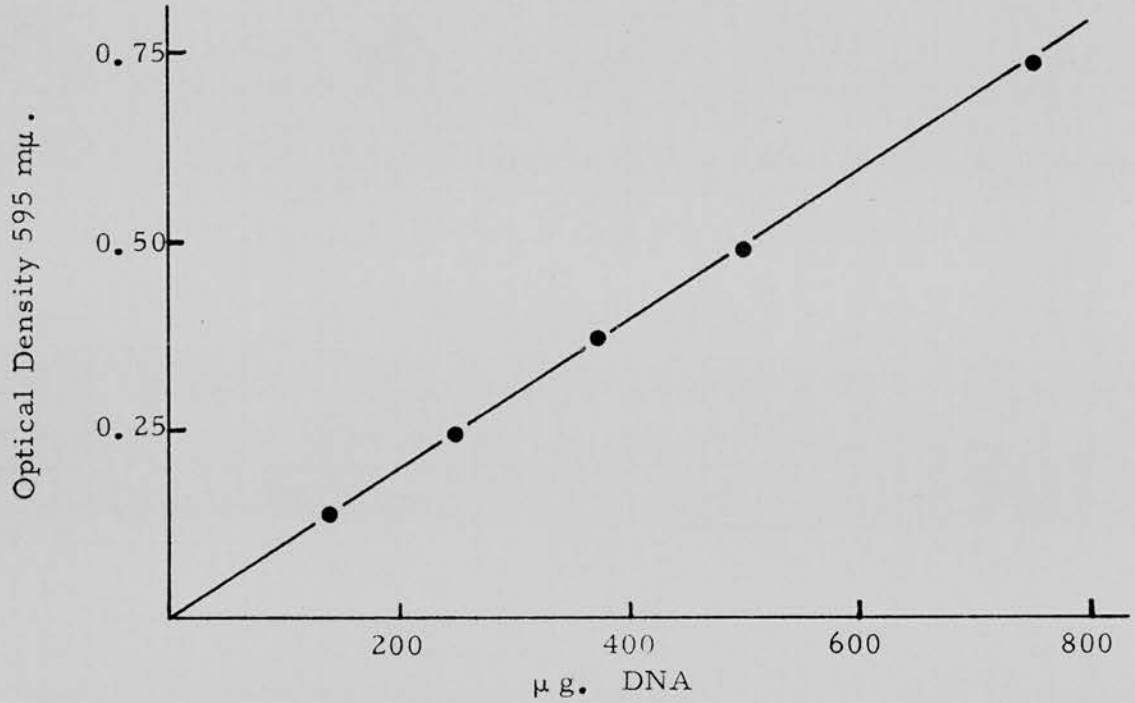
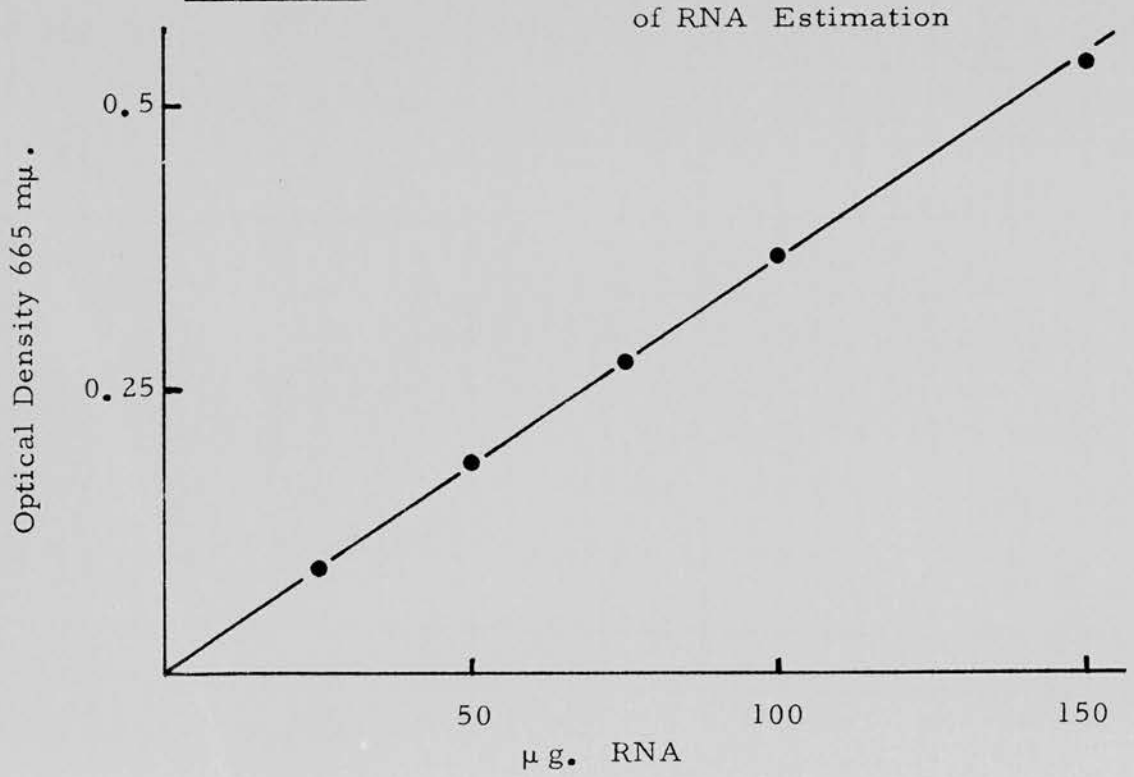


Figure 9 b.

Calibration Curve for the Orcinol Method of RNA Estimation



Determination of DNA

The diphenylamine reagent was prepared by dissolving 1 g. of diphenylamine (twice recrystallized from 70% ethanol) in 100 ml. "analar" glacial acetic acid, and then adding 2.75 ml. "analar" sulphuric acid.

A standard DNA solution containing 500 µg./ml. was prepared using herring sperm DNA (L.Lights and Co., Ltd., Colnbrook). The DNA was weighed out after drying *in vacuo* over P_2O_5, and dissolved in 0.5M perchloric acid.

1.5 ml. aliquots of the perchloric acid extracts, containing 50 to 750 µg. DNA, were measured into test tubes, and 3 ml. of the reagent added. After shaking, the tubes were heated in a boiling water bath for 10 min. The solutions were then cooled in tap water, and the optical densities of the blue colours read at 595 mµ. against water in a Unicam SP500 spectrophotometer. At least two reagent blanks and a series of standards were run simultaneously. Calibration curves for the estimation (Fig. 9 a) were found to be linear over the range 0 to 750 µg. DNA.

Determination of RNA

The orcinol reagent was prepared by dissolving 100 mg. $FeCl_3.6H_2O$ in 100 ml. HCl (S.G. 1.18) and adding 3.5 ml. of a 6% solution of orcinol (B.D.H.) in ethanol.

A standard solution of 100 µg. RNA/ml. in 0.5M perchloric acid was prepared from yeast RNA (C.F. Boehringer und Soehne, Gmbh., Mannheim) dried in vacuo over P_2O_5.

1.5 ml. aliquots of the perchloric acid extracts containing 10 - 150 µg. RNA were measured into test tubes, and 3 ml. of the reagent added. The reaction mixtures were heating in a boiling water bath for 3 min. and then cooled in tap water. The optical densities of the green solutions were then read at 665 mµ. against water in the spectrophotometer. Reagent blanks and standards were estimated simultaneously. Calibration curves were linear over the range 0 to 150 µg. RNA (Fig. 9 b).

Both the diphenylamine reaction for DNA and the orcinol reaction for RNA are susceptible to the presence of interfering substances in the perchloric acid extracts (Hutchison and Munro, 1961). Interference occurs particularly with the orcinol reaction which tends to give high values in the presence of various sugars and DNA. DNA gives approximately 12% of the colour obtained by the same weight of RNA, and this must therefore be taken into consideration when RNA is estimated in the presence of relatively large amounts of DNA.

In this work the estimations of DNA and RNA on whole organisms were expected to be less accurate than those carried out on subcellular components where less interfering substances (particularly of cell wall origin) would be present. However, in no case have any firm conclusions been drawn by assuming the values obtained to be highly accurate. Rather the values have been used as an indication of the amount of the nucleic acids present in a particular preparation, for comparison to the amount of basic protein also present.

THE BASIC PROTEINS OF BACTERIA

I. Introduction

The failure of various workers to isolate histone from bacterial DNA-protein preparations (see pp. 8 - 10) indicates that histone-like proteins may be completely absent from bacteria. The initial experiments were therefore performed to determine if any basic protein could be extracted from whole bacteria. As this was successful, further experiments were designed to characterise the proteins more fully, and to investigate their situation within the bacterial cell.

The basic proteins of Staphylococcus aureus, Micrococcus lysodeikticus, Bacillus megaterium, and Escherichia coli have been studied. All bacteria were cultured in the Department of Bacteriology, University of Edinburgh, and were gifted by Dr. J.F. Wilkinson, Dr. I.W. Sutherland and Dr. A.L.S. Munro.

II. Basic Proteins Isolated from Whole Bacteria

i. Preliminary Investigation and Development of Method for Purifying Basic Proteins (Staphylococcus aureus).

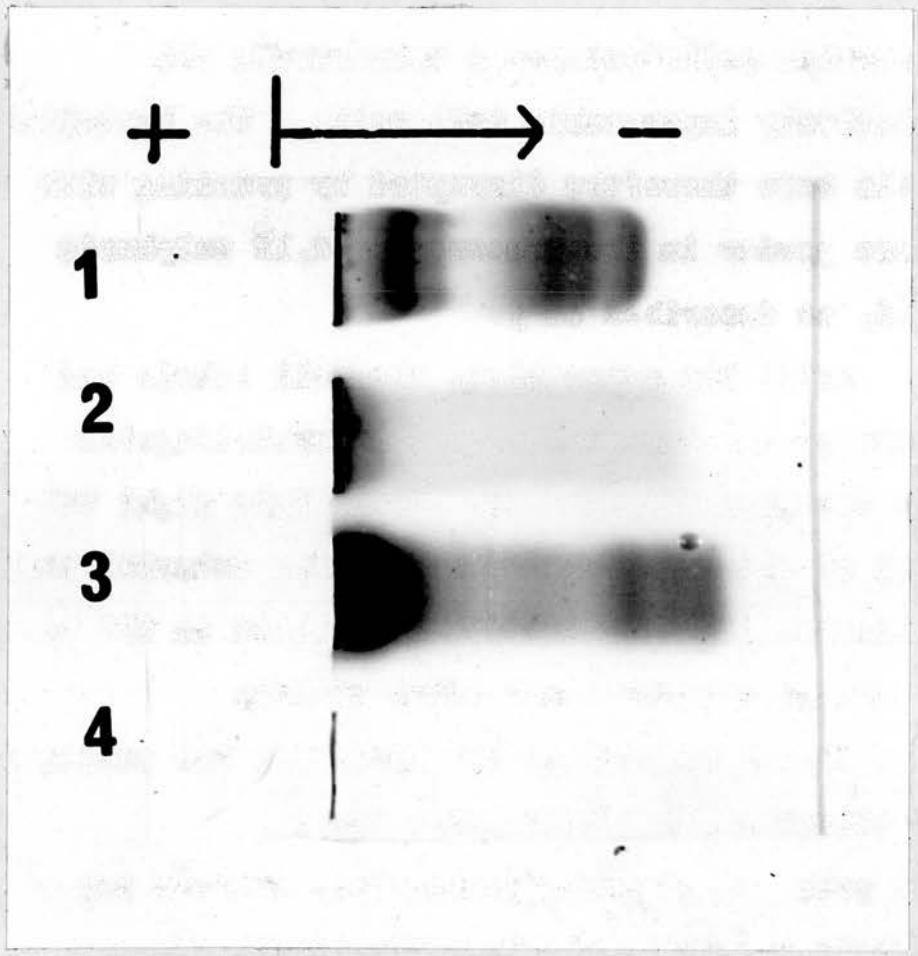
The first preliminary attempts to extract and purify basic protein from bacteria were performed on Staphylococcus aureus. These experiments led to the general method, as described on pp. 22-27 for the isolation of basic proteins from micro-organisms.

The work described in this section was the subject of a letter published in "Nature", (Cruft and Leaver, 1961). This paper is reprinted in the Appendix.

(a) Extraction In order to keep the conditions of extraction as close as possible to those used for the extraction of histone from thymus nuclei, the bacteria were washed once with 4% acetic acid, three times with 1% acetic acid and defatted and dried with ethanol and ether washes (three of each). The acetic acid washes, while ensuring a fairly acid pH, will also remove any remaining culture medium. The ethanol and ether treatment will denature many non-basic proteins, so rendering them insoluble to the subsequent acid extraction.

Figure 10.

Starch gel electrophoresis



1. Unfractionated calf thymus histone

2. Material extracted from S. aureus by 0.1$\bar{N}$ H_2SO_4 and precipitated by ethanol

3. Material from S. aureus after dialysis

4. Dialysate

Direct acid extraction of the intact bacteria was found to be inefficient, presumably because bacterial cells possess a very strong and relatively impermeable cell wall. The bacterial cells were therefore disrupted by grinding with glass powder in the presence of 0.1$\bar{N}$ sulphuric acid, as described on p. 23.

After the extraction, the cell debris and glass powder were sedimented by centrifugation and the clear supernatant poured into eight volumes of ethanol. The precipitated material was collected by centrifugation and dried in the usual manner with ethanol and ether washes.

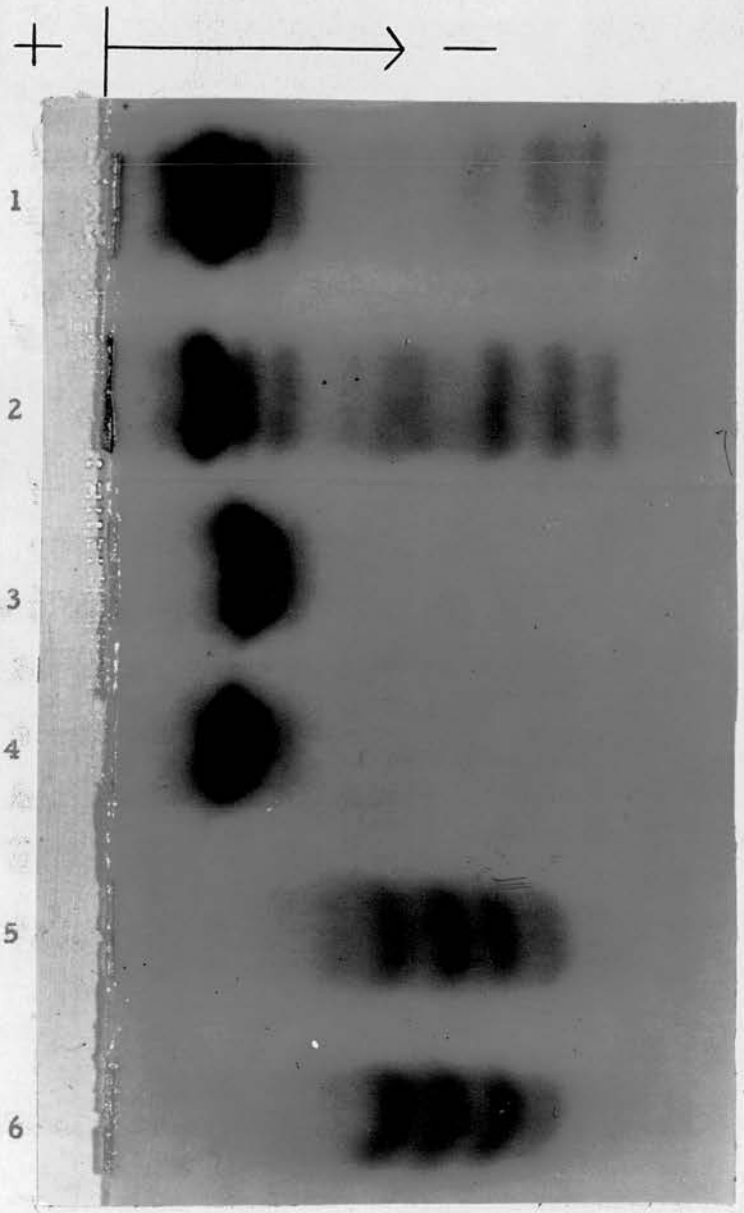
When a portion of this material was subjected to starch gel electrophoresis (pp. 28-33) the presence of protein migrating towards the cathode was detected (Fig. 10, sample 2). However, the intensity of staining was rather faint compared to that obtained with calf thymus histone, even although more of the bacterial material had been applied to the gel. It was considered that this indicated the presence of non-protein substances, possibly carbohydrate, which would be expected in an acid extract of whole bacteria.

(b) Effect of Dialysis The material was redissolved in 0.1$\bar{N}$ sulphuric acid and dialysed overnight against an equal volume of the acid. The solution outside the dialysis sac was poured into eight volumes of ethanol and a precipitate (the "dialysate" fraction) was obtained. The dialysis was continued for a further 24 hr. against large volumes of the acid. The "dialysed material" was then recovered from the dialysis sac by precipitation with ethanol, and dried in the usual way.

Samples of both the dialysed material and the dialysate were examined by starch gel electrophoresis, (Fig. 10, (3) and (4)). The dialysed material now gave more intensely staining bands, indicating that considerable purification of the protein had been achieved. The absence of protein in the dialysate fraction was concluded from the lack of any staining with Amido Black 10B. Experiments investigating the nature of the material removed by dialysis are described in section ii below.

Figure 11.

Starch gel electrophoresis showing the purification of S. aureus basic protein obtained using CM-cellulose



1, 2. Material extracted from S. aureus after dialysis and before application to CM-cellulose

3, 4. Non-basic fraction, not bound to CM-cellulose at pH 7.

5, 6. Basic fraction, bound to CM-cellulose at pH 7.

(c) Purification on CM-cellulose It was noticed during the electrophoresis of the dialysed material, that the rate of migration of the components with low mobilities was very dependent on careful pH adjustment of the sample before application to the gel. When the pH of the sample and gel were adjusted to above 6.5, the migration of these components was reversed, indicating that they are not basic in nature. Further purification of the basic proteins was therefore required.

Separation of the basic proteins with iso-electric points above pH 7, was attempted using a column of CM-cellulose. The procedure followed is described on pp. 24-27 Both the non-basic fraction passing directly through the column and the basic fraction subsequently eluted with 0.1$\bar{N}$ hydrochloric acid, were recovered by ethanol precipitation.

Starch gel electrophoresis of these two fractions (Fig. 11) indicated the complete separation of the non-basic slow mobility components, from the faster components with iso-electric points above pH 7.

Using the two purification steps, dialysis and separation on CM-cellulose, after extracting 16 g. of dry defatted bacteria three times with acid, 19 mg. of basic protein was obtained (i.e. 0.12% w/w of the dry defatted bacteria).

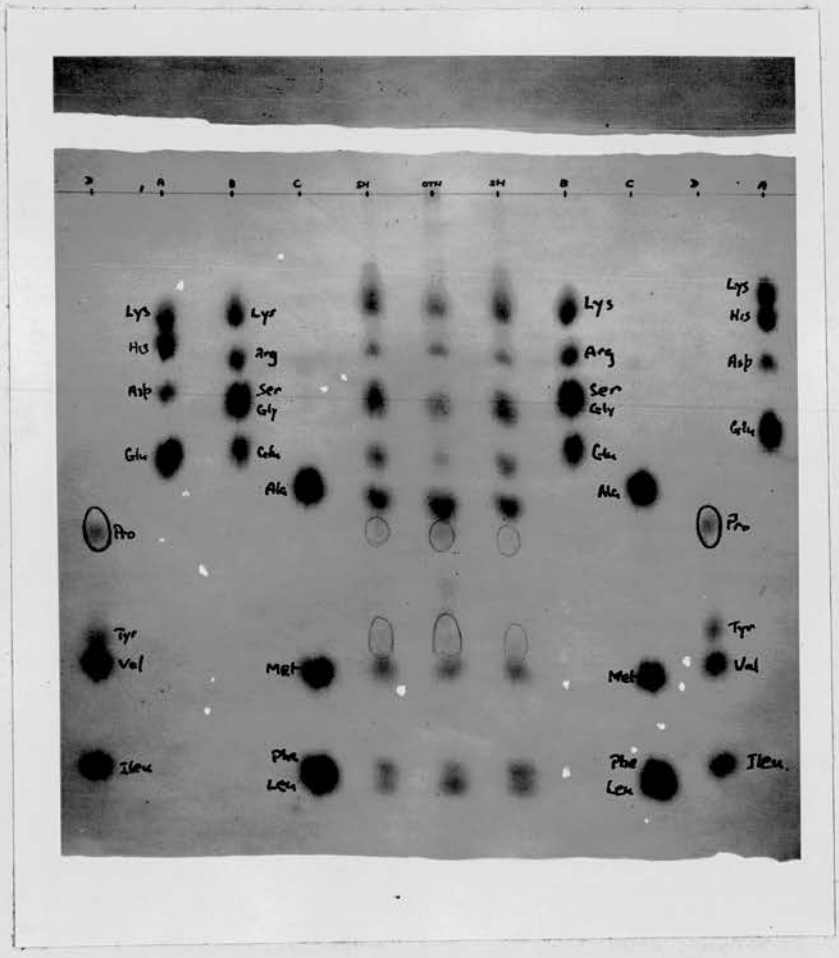
(d) Amino Acid Composition and Ultra-violet Absorption of the Basic Protein Isolated from S. aureus

In order to further characterise the purified basic protein fraction, the amino acids liberated on acid hydrolysis were studied by descending paper chromatography, and the ultra-violet absorption spectrum was plotted.

Equal quantities of the S. aureus protein and unfractionated calf thymus histone were hydrolysed with 6$\bar{N}$ hydrochloric acid under reflux, as described on p. 34 After running the chromatogram for 16 hr., it was dried and rechromatographed in the same direction, thus ensuring better separation of the amino acids with low R_F values.

Figure 12.

Chromatographic separation of amino acids in protein hydrolysates.



Spots developed with ninhydrin after two successive 16 hr. runs in one direction.

Chromatography paper: Whatman No. 1 (46 x 57 cm)

Solvent system: n-butanol:acetic acid:water = 63:27:10 by volume

Ninhydrin spray: 0.5 g. ninhydrin in 120 ml. n-butanol and 80 ml. acetone

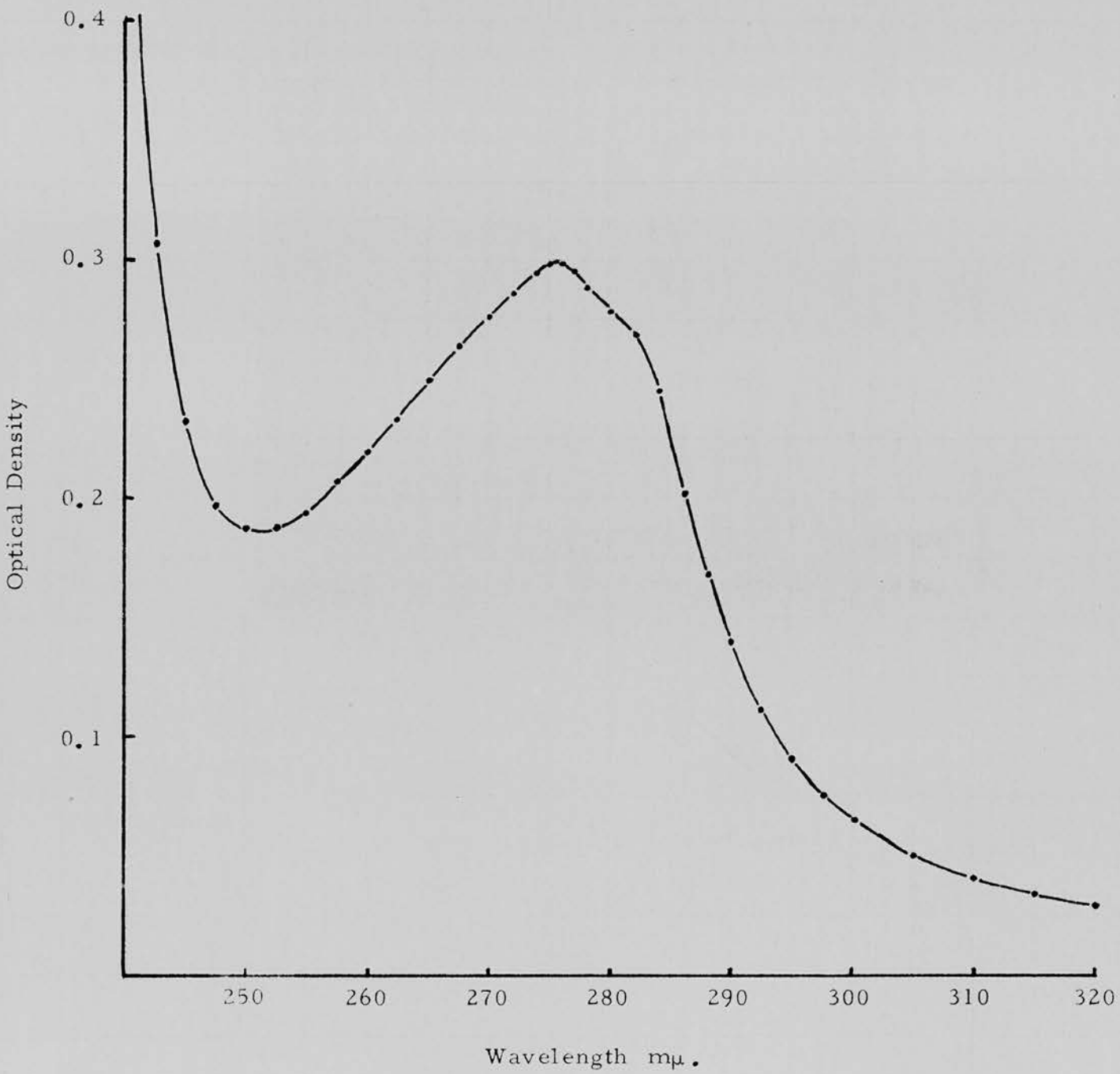
Hydrolysates
- OTH = Ox thymus histone
- SH = Basic protein fraction of S. aureus

Standard mixtures
- A = Lysine, histidine, aspartic acid and glutamic acid
- B = Lysine, arginine, serine, glycine and glutamic acid
- C = Alanine, methionine, phenylalanine, and leucine
- D = Proline, tyrosine, valine, and isoleucine

Figure 13.

Ultra-violet Absorption Curve of Basic Protein
From Staphylococcus Aureus

0.08% w/v solution. 0.5 cm. light path.



A photograph of the ninhydrin developed chromatogram is shown in Fig. 12. There is a clear similarity between the patterns of spots obtained with the two hydrolysates, indicating that the bacterial basic protein has a similar amino acid composition to calf thymus histone. Both proteins appear to contain relatively large amounts of lysine, arginine, alanine, proline and leucine, with smaller amounts of histidine, tyrosine, and the acidic amino acids.

The ultra-violet absorption curve of the S. aureus protein is shown in Fig. 13. This is very similar to the curve for calf thymus histone (Fig. 8), and hence indicates the presence of tyrosine, but not tryptophan, in the bacterial protein. The curve also shows that the S. aureus protein is free from contamination with nucleic acid, which has an absorption maximum at 260 mμ.

These results led to the conclusion that the basic proteins isolated from S. aureus are apparently quite similar to the histones of higher organisms.

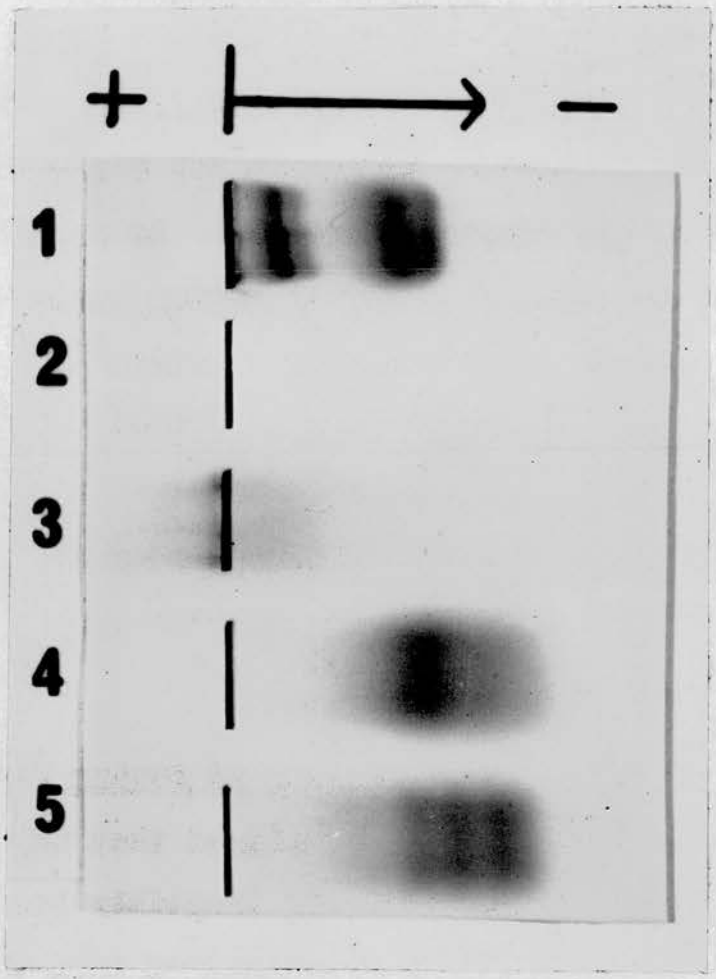
ii. Isolation of Basic Protein from Micrococcus lysodeikticus, and Investigation of the Material Removed during the Purification

In order to confirm that the extraction and purification of basic protein as described for S. aureus could be applied to other species of bacteria, these procedures were repeated on the bacteria Micrococcus lysodeikticus. The nature of the material removed during the two purification steps was also investigated.

(a) Isolation of M. lysodeikticus Basic Protein The bacteria were washed with dilute acetic acid, and defatted and dried with ethanol and ether. The basic proteins were then extracted from the dry bacteria, and purified by dialysis and separation on CM-cellulose. All these operations were carried out as described for the S. aureus bacteria. The material removed by the first dialysis was recovered by precipitation with ethanol, and dried with ethanol and ether. Similarly the non-basic material not bound to the CM-cellulose column was recovered from the eluate.

Figure 14.

Starch gel electrophoresis



1. Calf thymus histone
2. M. lysodeikticus - dialysate
3. M. lysodeikticus - non-basic fraction, not bound to CM-cellulose at pH 7
4. M. lysodeikticus - basic protein, bound to CM-cellulose at pH 7
5. S. aureus basic protein

From 6.5 g. of dry bacteria, 3.3 mg. of basic protein was obtained, representing a yield of 0.05%. Both the basic protein and the non-basic material removed by the CM-cellulose separation, were examined by starch gel electrophoresis (Fig. 14, samples 3 and 4). As with the material isolated from the S. aureus bacteria the stained gel showed complete separation of components with lower electrophoretic mobilities from the faster moving basic components. The absence of protein in the material removed by dialysis was again indicated by its lack of staining (Fig. 14, sample 2).

(b) Nature of Material Removed During Purification At first it was considered that considerable amounts of carbohydrate (possibly containing amino sugars) might be extracted from the bacteria by the 0.1$\bar{N}$ sulphuric acid, and that this was the material removed by dialysis. In an attempt to confirm this, quantitative sugar reactions were carried out on both the material removed by dialysis and the non-basic material removed by the CM-cellulose separation. The results obtained led to further investigation of these substances, and particularly of the material removed by dialysis.

Carbohydrate was estimated by the modified Molisch Reaction as described by Dische (1955). The intensities of the purple colours were compared visually to the intensities obtained with standard sucrose solutions. The results are given in Table 3.

TABLE 3

Carbohydrate contents determined as sucrose by the modified Molisch Reaction.

Material investigated	Carbohydrate estimate as µg. sucrose/mg.	% of carbohydrate in material
Material removed by dialysis	8 µg.	0.8%
Material removed by CM-cellulose separation	200 µg.	20%
Purified basic protein from *M. lysodeikticus*	trace	< 0.2%
Calf thymus histone	trace	< 0.2%

Rather surprisingly these results indicated the presence of little carbohydrate (less than 1%) in the material removed by dialysis. However, a considerable portion of the non-basic material

removed during the CM-cellulose separation was carbohydrate in nature, and the effectiveness of this procedure in removing the carbohydrate impurity was indicated by the very low carbohydrate content of the purified basic protein. Analysis of the basic protein isolated from S. aureus also showed freedom from carbohydrate.

The low carbohydrate content of the dialysate material was confirmed by the anthrone reaction (Dische, 1955), which gave a value of 0.2% carbohydrate (estimated as glucose). Also, estimation of amino-hexose by reaction with acetylacetone and p-dimethylaminobenzaldehyde as described by Rondle and Morgan (1955), using glucosamine as standard, showed only the presence of traces of amino-hexose.

The ultra-violet absorption curve of a solution of the dialysate material had a maximum absorption at 256 mμ, indicating the presence of nucleotides. However, it was calculated that, even if all the absorption at this wavelength was due to nucleotides, these nucleotides would account for well under 1% of the total material present. Further evidence that protein was not present in the material, already indicated by the lack of

any staining after starch gel electrophoresis, was given by the absence of any shoulder on the absorption curve at 280 mμ.

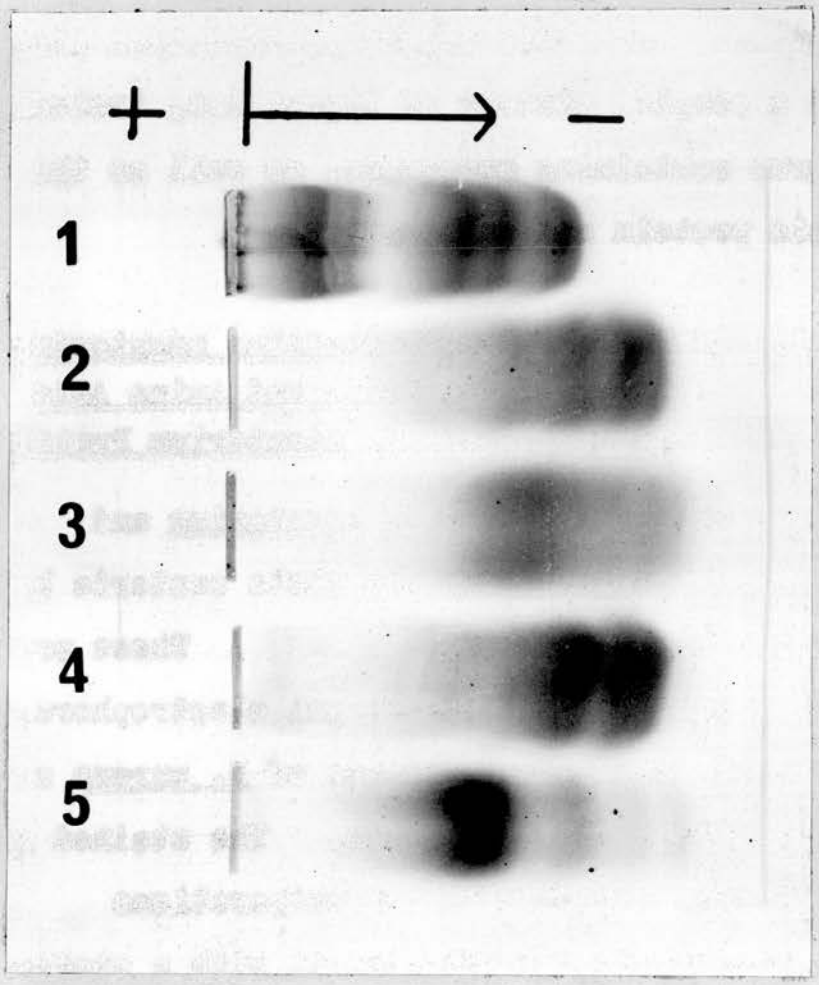
A sample of the dialysate material was hydrolysed with 6$\bar{N}$ hydrochloric acid at 100°C for 24 hr. Paper chromatography of the hydrolysate failed to show the presence of any amino acids, thus confirming the absence of protein.

Quantitative analysis of the dialysate material showed that it contained only 1.48% carbon and 1.99% hydrogen. However, 78.7% of non-combustible residue was obtained. These results indicated that the material was largely inorganic in nature.

Qualitative tests for phosphate showed the presence of a little free orthophosphate. After hydrolysis of the material with 1$\bar{N}$ sulphuric acid for 1 min. at 100°C, the phosphate test became strongly positive. Estimation of the total phosphorus content by the method of Allen (1940) gave a value of 4.3%. Thus a considerable portion of the dialysate material (about 20%) appears to be some form of phosphate - possibly the polyphosphate which has been reported as occurring in the volutin granules of some bacteria (Widra, 1959).

Figure 15.

Starch gel electrophoresis of calf thymus histone and basic proteins isolated from whole bacterial cells



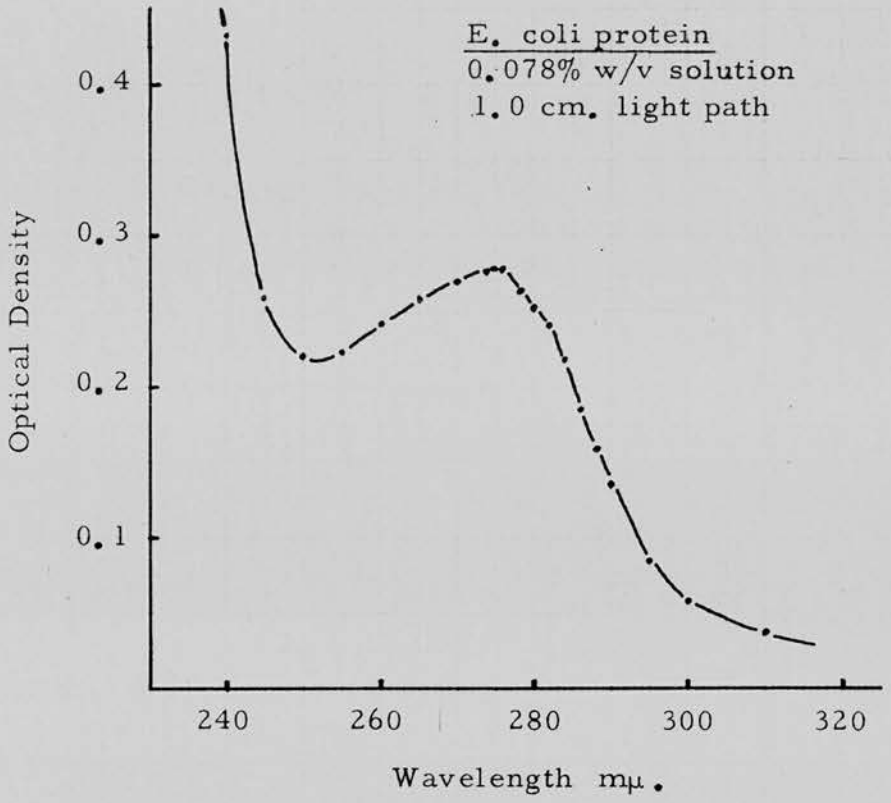
1. Calf thymus histone
2. S. aureus basic protein
3. M. lysodeikticus basic protein
4. B. megaterium basic protein
5. E. coli basic protein

Phosphorus (1.4%) was also found in the non-basic material obtained after the CM-cellulose separation. Thus this second purification step removes a complex mixture of impurities, including phosphorus containing compounds, as well as the non-basic protein and carbohydrate.

iii. Basic Proteins of Bacillus megaterium and Escherichia coli, and Amino Acid Analysis of the B. megaterium Protein

The basic proteins of *B. megaterium* and *E. coli* were isolated from the whole bacteria by the routine procedure (pp. 22 - 27) These proteins were subjected to starch gel electrophoresis, together with the basic proteins of *S. aureus* and *M. lysodeikticus* for comparison. The stained gel (Fig. 15) shows that all four preparations contain several protein components with a similar range of mobilities. The fastest moving bands have mobilities which are slightly greater than the fastest components of calf thymus histone. The bacterial preparations show little staining at or close to the origin, indicating the absence of high molecular weight aggregated material as occurs in the calf thymus histone preparation. In this respect the bacterial proteins resemble the a histone fraction of calf thymus, rather than the unfractionated histone.

Figure 16. Ultra-violet Absorption Curves of Bacterial Basic Proteins



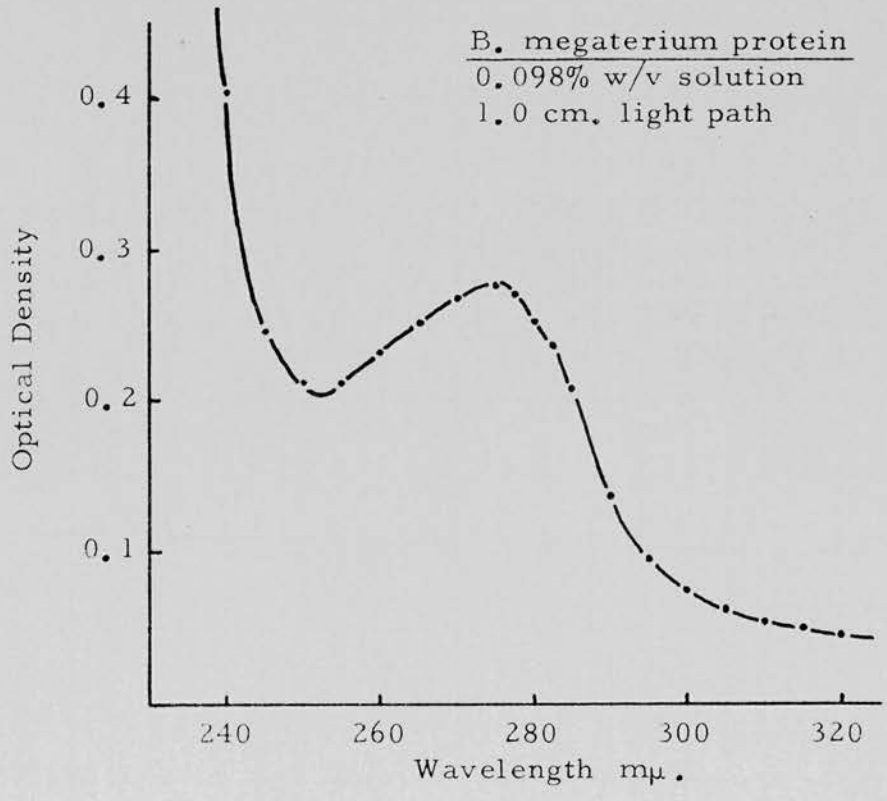


TABLE 4

The analysis of arginine, lysine, glutamic acid, aspartic acid, and tyrosine residues in B. megaterium basic protein, compared to data for unfractionated calf thymus histone, and the β fraction

All values given as g. amino acid residue in 100 g. protein

Amino acid residue	B. megaterium basic protein	Unfractionated calf thymus histone*	β fraction, calf thymus histone**
Arginine	11.4 (11.6, 11.6, 11.0)	10.3	16.1
Lysine	14.2 (13.2, 14.7, 14.7)	15.5	9.9
Glutamic acid	8.1 (7.8, 8.3, 8.3)	8.5	10.7
Aspartic acid	9.0 (9.3, 9.7, 7.9)	4.4	5.7
Tyrosine	(minimum value) 1.9	3.3	3.8
Arg + Lys	25.6	25.8	26.0
Glu + Asp	17.1	12.9	16.4
$\frac{\text{Arg + Lys}}{\text{Glu + Asp}}$	1.50	2.00	1.58

* Data of Crampton et al. (1955) converted to g. amino acid residue in 100 g. protein

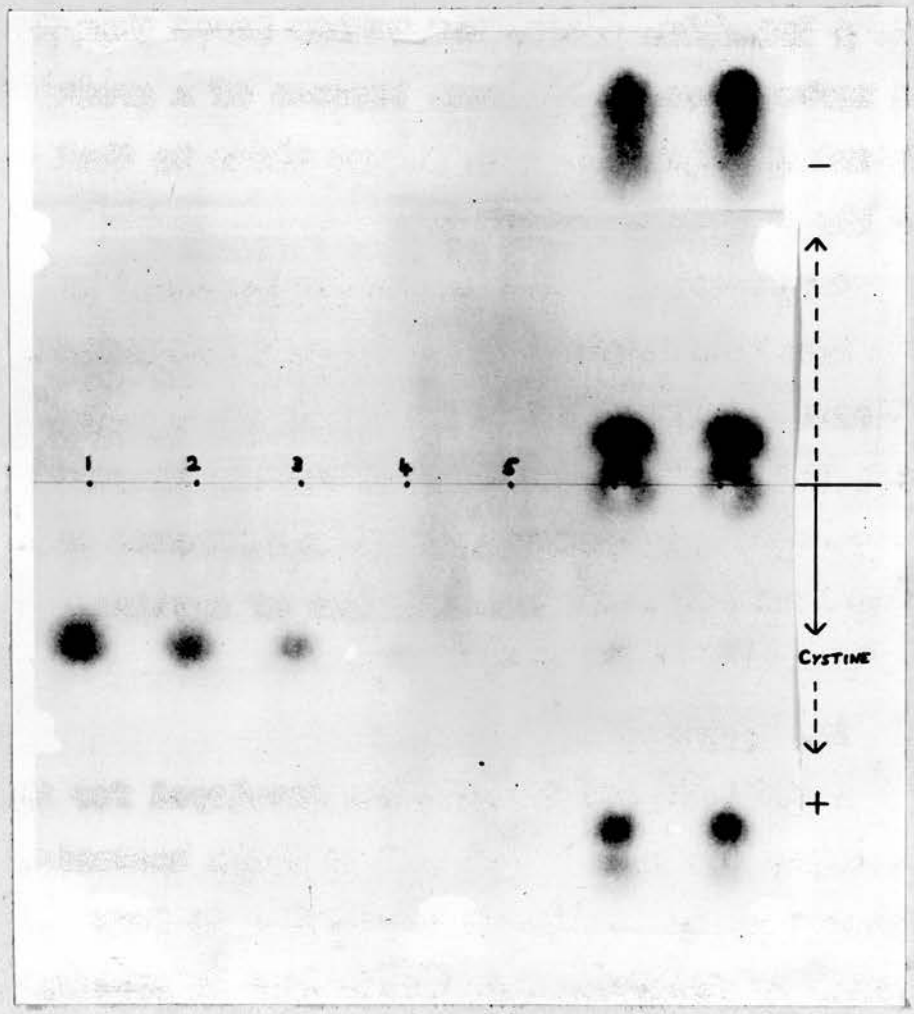
** Data of Cruft et al. (1958).

The ultra-violet absorption curves of both the B. megaterium and E. coli preparations are given in Fig. 16. These show the characteristic histone-type curve, with maximum absorption at 276 mμ, although the non-specific background absorption is higher than normally obtained with histone preparations. These curves are again taken as indicating the presence of tyrosine, but not tryptophan, in the bacterial proteins.

A sample of the B. megaterium basic protein was hydrolysed and the arginine, lysine, glutamic acid, and aspartic acid in the hydrolysate were estimated after electrophoretic separation by the ninhydrin-copper method. The tyrosine content of the protein (minimum value) was estimated from the absorption curve. The amounts of these amino acids in the protein were then calculated as g. amino acid residue per 100 g. protein. These results are given in Table 4, together with, for comparison, the data of Crampton et al.(1955) for unfractionated calf thymus histone, and of Cruft et al. (1958) for the β histone fraction.

Figure 17.

Electrophoretic separation of cystine in protein hydrolysates. Buffer - 0.05M Veronal pH 8.6 4.5 hr. at 10.0 volts/cm.



1 - 5 Cystine standards

6 Hydrolysate of B. megaterium basic protein

7 Hydrolysate of calf thymus histone

It was observed that the analyses of arginine, lysine and glutamic acid in the B. megaterium protein closely resembled the data for the unfractionated histone. Although the ratio arg. + lys./glu. + asp. was rather lower than for the unfractionated histone, because of a greater content of aspartic acid, it was close to that for the β histone fraction.

Examination of the B. megaterium basic protein hydrolysate for cystine by paper electrophoresis indicated the presence of trace amounts (Fig. 17). However, the simultaneous electrophoresis of a hydrolysate of unfractionated calf thymus histone also showed traces of cystine.

iv. Summary

A suitable method has been developed for the isolation of basic proteins from whole bacteria. This method was applied successfully to four species of bacteria, and should also be applicable to the preparation of basic proteins from other types of organism and from sub-cellular fractions.

Starch/

Starch gel electrophoresis of the bacterial basic protein preparations has demonstrated the presence of several components, with a range of mobilities similar to calf thymus histone.

Examination of the ultra-violet absorption curves, and paper chromatography of a hydrolysate of the S. aureus basic protein, indicated that, on the basis of amino acid composition, the bacterial proteins are very similar to the histones of higher organisms. This similarity was confirmed by the quantitative analysis of arginine, lysine, aspartic acid, glutamic acid, and tyrosine residues in the basic protein isolated from B. megaterium.

III. The Intracellular Distribution of Basic Proteins in Bacteria

i. Introduction

Having established the presence of basic proteins in whole bacterial cells, a series of experiments were performed to investigate the location of these proteins within the cell. In particular it was considered that these proteins might be associated with either DNA or RNA. Attempts were therefore made to extract basic proteins from both bacterial ribosomes and DNA-protein preparations.

Quantitative Estimation of DNA, RNA, and Total Basic Protein in Whole Bacteria

DNA and RNA in the whole bacteria were determined as described on pp. 52-55. These estimations on the whole organisms would be susceptible to inaccuracy due to the presence of interfering substances in the perchloric acid extracts, and therefore have only been taken as giving an indication of the order of amount of the nucleic acids present.

The total basic protein present in the whole organisms was estimated by repeating the acid extraction of the bacteria until no further basic protein was obtained. This required some six or seven extractions in all. The protein from all the extractions was then combined and weighed. The accuracy of these determinations would depend on the recovery of basic protein during the purification procedures; however, there has been no evidence of substantial losses at any stage.

Preparation of DNA-protein from Bacteria

The preparation of DNA-protein from bacteria was based on the method for the isolation of calf thymus nucleohistone using solutions of high ionic strength, as described by Jordan (1960). This method was adopted because of the failure of several workers to show the presence of histone in DNA-proteins isolated from bacteria by extraction with low ionic strength solutions (see p.9-10). On the other hand, Palmade et al. (1958) claimed to have isolated typical nucleohistone by a high ionic strength extraction procedure, (pp. 11-12).

In order to minimise enzymic degradation of the DNA-protein, all operations were carried out as rapidly as possible and at 0°C. This temperature was maintained by using refrigerated centrifuges, and by keeping all apparatus in crushed ice in the cold room. The use of citrate during the preparation is also claimed to suppress enzymic action (Jordan, 1960).

The harvested bacteria were washed twice in a centrifuge with approximately four volumes of an ice-cold solution containing 0.1$\bar{M}$ sodium chloride, 0.05$\bar{M}$ trisodium citrate, and previously adjusted to pH 7.0. After disintegration, by grinding with twice their packed weight of fine glass powder in a mortar for 30 min., the bacteria were extracted briefly by suspension in three volumes of the sodium chloride-citrate solution. After centrifuging at 25,000 x g. for 15 min. the DNA-protein was extracted from the sedimented material with 1.0$\bar{M}$ sodium chloride (two volumes per volume of the sedimented material). This extraction was carried out in the mortar for 24 hr., occasionally stirring with the pestle. The insoluble material and glass powder were removed by centrifuging twice at 25,000 x g. for 1 hr.

The DNA-protein was then precipitated by reducing the sodium chloride concentration of the supernatant to 0.15M. This was achieved by dialysing the solution overnight against a suitable volume of dilute sodium chloride. This method was used by Palmade (1961) as it ensures a higher yield than precipitation by simple dilution. The precipitated material was collected by centrifugation (25,000 x g. for 30 min.) and then purified by redissolving in a small volume of 1.0M sodium chloride and reprecipitating by dialysis. The final product, again collected by centrifugation, was dried by washing with ethanol and ether.

Basic protein was extracted from the DNA-protein with 0.1N sulphuric acid, and after precipitation with ethanol, was dried with ethanol and ether in the usual manner.

Isolation of Bacterial Ribosomes

The preparation of bacterial ribosomes was based on the method used by Tissières and Watson (1958) for the isolation of 100s ribosomes from E. coli. Fine glass powder was used instead of alumina to disrupt the bacteria, and the treatment with deoxyribonuclease was omitted.

The solution used for extracting the bacteria was 0.01M magnesium acetate in 0.001M tris buffer, pH 7.4. This solution was diluted to half concentration for further resuspensions of the ribosomes.

The harvested bacteria were washed twice in a refrigerated centrifuge with five volumes of the buffered magnesium acetate solution. The bacteria were then transfered to a mortar and three times their packed weight of fine glass powder was added. This mixture was ground vigorously for 30 min. in the cold room, and the disintegrated cells were then extracted by suspending in five volumes of the buffer solution.

In order to remove the glass powder and cell debris, the extract was centrifuged twice at 12,000 x g for 15 min. The supernatant was then centrifuged at 105, 000 x g for 180 min. in a "Spinco" Model L centrifuge (rotor No.30). The sedimented ribosomes were resuspended in buffered magnesium acetate solution of half concentration. After a preliminary centrifugation at 12,000 x g for 15 min., the ribosomes were resedimented by centrifuging at 145,000 x g for 120 min.(rotor No. 40). The ribosomes were finally defatted and dried by washing with ethanol and ether.

Basic proteins were extracted from the ribosomes with 0.1$\bar{N}$ sulphuric acid, and purified by dialysis and separation on CM-cellulose. This purification was performed exactly as described for the isolation of basic proteins from the whole bacteria.

ii. The Basic Proteins of B. megaterium

Analysis of the whole B. megaterium bacteria the following values, given as percentages of the dry defatted bacteria:-

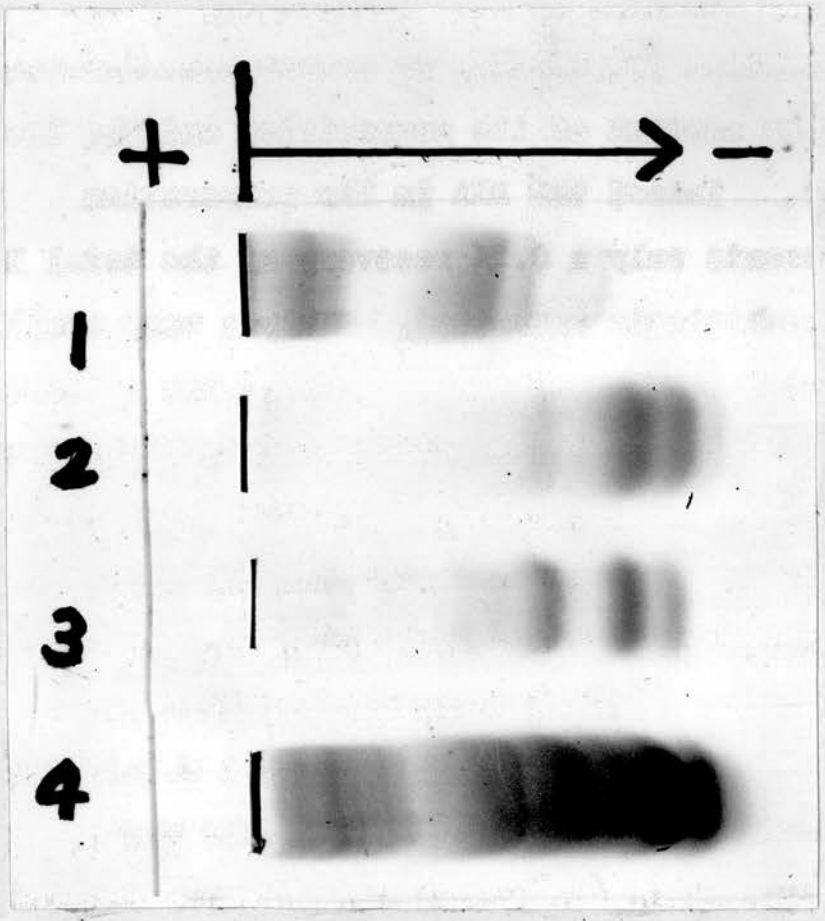
0.32% basic protein; 3.7% DNA; and 13.0% RNA.

Although these figures can only be taken as approximate, they immediately indicate that the bulk of the DNA in the bacterial cell does not exist as a nucleohistone complex of the type found in the nuclei of higher organisms, where there are approximately equal quantities of DNA and histone (Phillips, 1962).

The attempt to isolate DNA-protein from B. megaterium gave a very low yield. The material obtained only represented 0.1% of the dry mass of the bacteria extracted. Analysis of this material showed that it contained approximately

Figure 18

Starch gel electrophoresis



1. Calf thymus histone
2. B. megaterium - basic protein isolated from whole bacteria
3. B. megaterium - basic protein isolated from ribosomes
4. B. megaterium - basic protein isolated from DNA-protein preparation

9% DNA, 2% RNA and 10% of the non-purified basic protein isolated by acid extraction. Thus this DNA-protein preparation is rather unsatisfactory both on account of its composition and the low yield. Indeed the DNA in the preparation represents only a 0.2% recovery of the total DNA of the bacteria extracted. Only a very small amount of basic protein (< 1 mg.) was obtained and its further purification on CM-cellulose was therefore not attempted. However there was sufficient material to carry out one electrophoresis run in starch gel (Fig. 18, sample 4). This gave a complex pattern of protein bands which was quite different from that obtained with the basic protein extracted from the whole bacteria (Fig. 18, sample 2). It was considered that the slower components might be non-basic in nature, and would have been removed if a CM-cellulose separation had been employed. The numerous faster components could be quantitatively minor components of the total basic protein of the organism, thus explaining the failure to detect all these bands in the material extracted from the whole bacteria.

The preparation of ribosomes from B. megaterium presented no difficulty. The yield of ribosomes represented almost 4% of the initial bacterial dry mass, and analysis showed them to contain 2.0% CM-cellulose purified basic protein and 64% RNA. No DNA was detected in the ribosomes by the diphenylamine reaction.

The basic protein obtained from a single ribosomal preparation represented 0.077% of the bacterial dry mass from which these ribosomes had been prepared. As the bacteria contain 0.32% basic protein, this indicates that at least 24% of the basic protein in these bacteria is ribosomal. Since the ribosomes obtained in a single preparation must only be a portion of the total, because of incomplete disintegration and extraction of the bacteria, it was concluded that a much higher percentage of the total bacterial basic protein must be ribosomal.

Starch gel electrophoresis of the ribosomal basic protein showed the presence of similar protein components in both this material and in the basic protein isolated from the whole organisms (Fig. 18, samples 2 and 3). This was taken as further evidence for the conclusion that the bulk of the basic protein in B. megaterium is ribosomal in origin.

iii. The Basic Proteins of E. coli

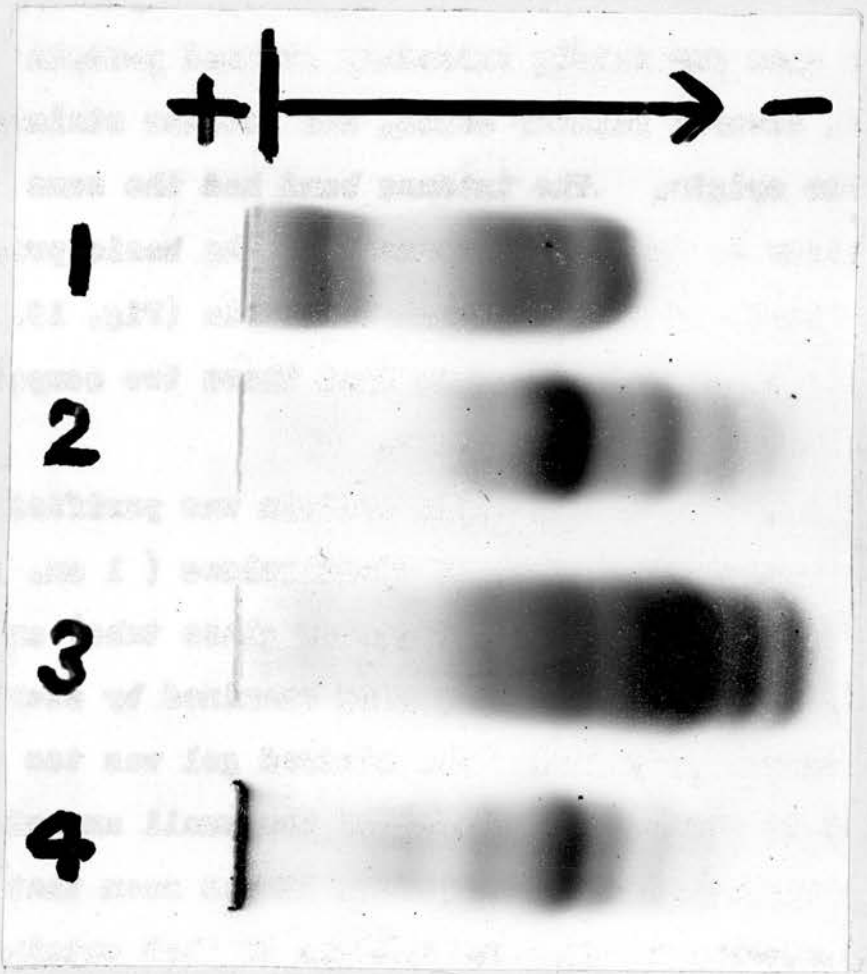
Because the DNA-protein preparation from B. megaterium was rather unsatisfactory, the experiments examining the intracellular distribution of bacterial basic proteins were repeated on a second species. E. coli was chosen as it was from this species that Palmade et al. (1958) had claimed to have isolated a typical nucleohistone complex.

The whole E. coli bacteria were found to contain approximately 0.96% total basic protein, 7.1% DNA and 12.5% RNA. These results indicate, as did those on whole B. megaterium, that a large proportion of the bacterial DNA can not be in the form of a typical nucleohistone complex.

The DNA-portein preparation obtained from the E. coli represented 0.21% of the dry mass of the bacteria extracted. Analysis of this material showed that it contained approximately 22.4% DNA, 1.6% RNA and 8.7% of the acid-extracted basic protein (not purified further). Therefore, on the basis of both yield and composition, this DNA-protein preparation is only a slight improvement on that extracted from the B. megaterium. In this case the recovery of DNA in the DNA-protein preparation is only 0.6% of the total DNA in the bacteria extracted.

Figure 19.

Starch Gel Electrophoresis



1. Calf thymus histone
2. E. coli - basic protein isolated from whole bacteria
3. E. coli - Basic protein isolated from ribosomes
4. E. coli - Basic protein isolated from DNA-protein preparation

A portion of the basic protein extracted from the DNA-protein preparation was subjected to starch gel electrophoresis (Fig. 19)(sample 4). This gave one fairly intensely stained protein band, several fainter bands, and intense staining at the origin. The intense band had the same mobility as the main component of the basic protein isolated from the whole bacteria (Fig. 19, sample 2), thus indicating that these two components have the same identity.

A little of the basic protein was purified on a very small column of CM-cellulose (1 cm. of CM-cellulose in a 4 mm. diameter glass tube) and the purified material was also examined by starch gel electrophoresis. The stained gel was too faint to photograph because of the small amount of protein applied. However, it was seen that the pattern of bands was similar to that obtained with the unpurified protein, except that there was no longer any staining at or close to the origin. The non-migrating protein in the unpurified preparation is therefore apparently not basic.

The ribosomes obtained from E. Coli in a single preparation represented 3.0% of the initial bacterial dry mass. These ribosomes were found to contain 2.4% CM-cellulose purified basic protein, 38.2% RNA, and 2.1% DNA. Thus, unlike the B. megaterium ribosomes, the E. coli ribosomes are contaminated with some DNA. This could presumably be avoided if deoxyribonuclease was utilised during the preparation, as suggested by Tissières and Watson.

The purified basic protein obtained from the ribosomal preparation was 0.072% of the dry mass of the bacteria from which the ribosomes were prepared. As the bacteria contain 0.96% total basic protein, this indicates that at least 7.5% of the total basic protein is ribosomal. However, as discussed in the case of the B. megaterium experiments, the ribosomal basic protein may account for a much higher proportion of the total basic protein than this minimum value.

Starch gel electrophoresis of the ribosomal protein (Fig. 19, sample 3) gave a rather similar pattern of stained bands to that obtained with the total basic protein extracted from the whole

bacteria (Fig. 19, sample 2). However there was some difference in the relative intensities of the bands, with the faster components of the ribosomal preparation appearing more intense. This indicates that the ribosomal basic protein and the total basic protein do not contain the same proportions of the different components separated by electrophoresis, and therefore it is unlikely that all the basic protein of E. coli is ribosomal in origin.

iv. The Association of E. coli DNA with Calf Thymus Histone

The unsatisfactory nature and poor yield of the bacterial DNA-protein preparations would be accounted for if the greater part of the bacterial DNA is free from association with histone-like protein. Indeed this was indicated by the estimations of DNA and total basic protein on the whole bacteria. However it was also considered that the structure of bacterial DNA might differ from vertebrate DNA in such a way that the bacterial DNA-histone, if present, would not have the same solubility characteristics, and hence a preparative procedure based on that for vertebrate DNA-histone would be unsatisfactory.

At attempt was therefore made to demonstrate that a complex between E. coli DNA and calf thymus histone exhibits similar properties in salt solutions to mammalian DNA-protein.

During the attempt to isolate E. coli DNA-protein, the molar salt extract was dialysed to reduce the salt concentration to 0.15$\bar{M}$, and the precipitated material was collected by centrifugation (p. 75). As recovery of the total DNA of the bacteria in the DNA-protein preparation was less than 1%, the clear viscous supernatant presumably still contained the bulk of the bacterial DNA. This supernatant was collected, and sufficient sodium chloride was added to increase the salt concentration to 1.0$\bar{M}$. Unfractionated calf thymus histone (2.5 mg./ml.) was stirred into the solution until dissolved. The solution was then dialysed overnight against a suitable volume of dilute sodium chloride solution in order to reduce the salt concentration to 0.15$\bar{M}$. A large amount of material was precipitated, and this was collected by centrifugation and dried by washing with ethanol and ether. Analysis of this material showed that it contained approximately 35% DNA, 1.7% RNA, and 25% of basic

protein (purified on CM-cellulose). On subjection to starch gel electrophoresis the basic protein was found to give the typical calf thymus histone pattern of stained bands. Hence this basic protein recovered is apparently the histone which was added, and the bulk of the material precipitated at 0.15M salt concentration appears to be bacterial DNA complexed with calf thymus histone. This complex also exhibits the same solubility properties in salt solutions as vertebrate DNA-histones, as it is insoluble in 0.15M sodium chloride but soluble at a 1M concentration. The DNA in the material precipitated represented approximately 35% of the total DNA present in the bacteria initially extracted, and 25% of the histone which had been added was recovered from this material.

This experiment indicates that, if DNA were complexed with a histone-like protein in the bacterial cell, then a good yield of DNA-protein would be expected by the isolation procedure that was attempted. The failure to obtain a good yield of DNA-protein therefore appears to be because most of the DNA in bacteria is free ffom combination with basic protein, and not because some difference in the composition of the bacterial DNA is altering the solubility characteristics of a DNA-basic protein complex.

v. Summary and Discussion

By studying the total content of DNA and basic protein in bacteria it was shown that only a small portion of the DNA(< 10%) could be in the form of a typical nucleohistone complex. Further experiments indicated that a large proportion of the total basic protein which can be extracted from bacteria must be ribosomal in origin. Both B. megaterium and E. coli ribosomes were found to contain approximately 2% of basic protein.

The attempts to isolate DNA-protein from bacteria gave very low yields, the recovery of the total bacterial DNA always being well below 1%. The DNA-protein preparations were also unsatisfactory because, even after reprecipitation, DNA and basic protein together accounted for less than 30% of their composition. These results appear to confirm that a large portion of the DNA in the bacterial cell must be free from association with basic protein of the histone type.

The possibility still exists that a small portion of the DNA in bacteria could be complexed with basic protein and that such a DNA-protein is recovered in the isolation procedure attempted. However, it seems just as probable that the basic

protein in the DNA-protein preparation is from some other origin in the cell, and becomes associated with the DNA during the extraction procedure.

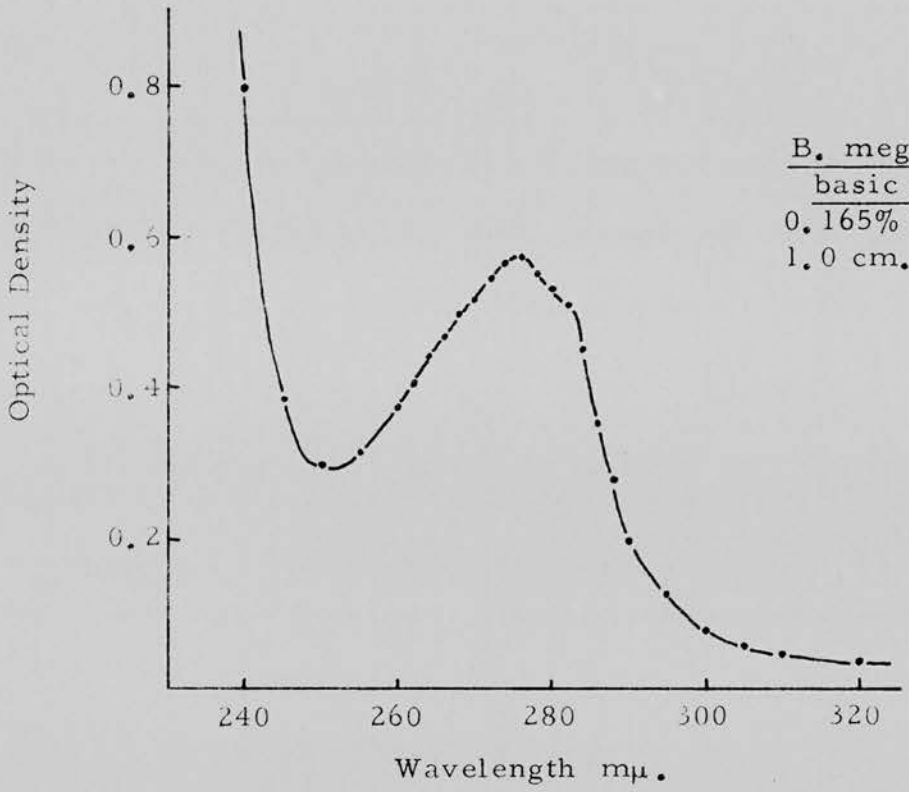
IV. Fuller Characterisation of the Ribosomal Basic Protein of Bacteria

i. Introduction

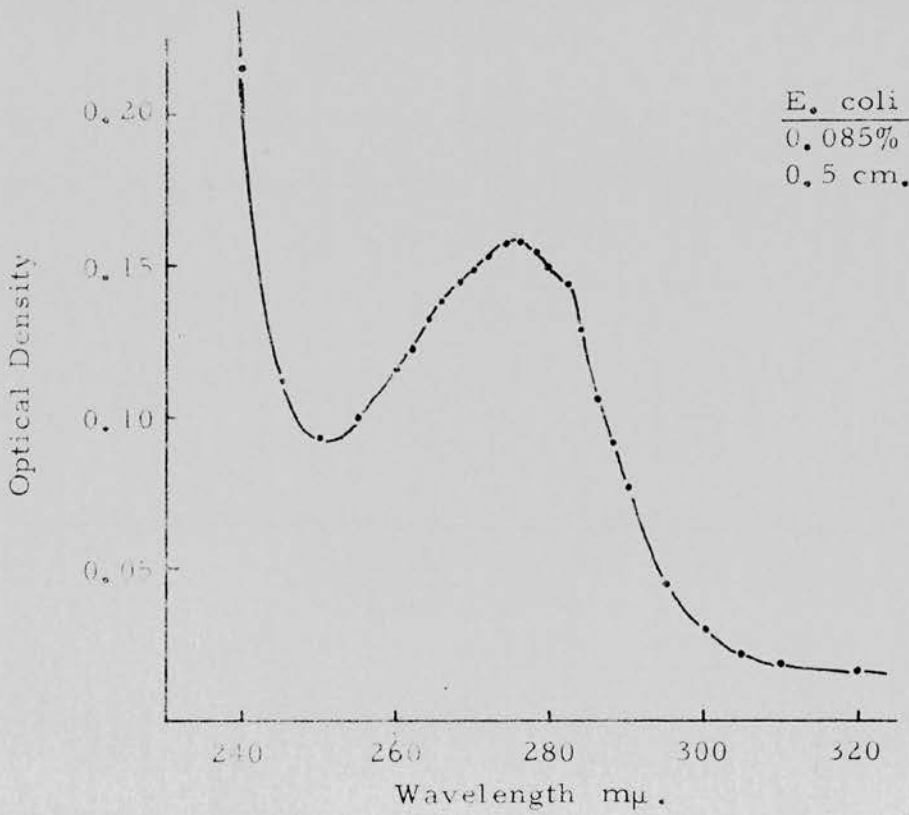
The basic proteins isolated from whole bacteria are apparently very similar to the histones of higher organisms (p. 71). As a large portion of the total basic protein of both B. megaterium and E. coli is ribosomal, it is to be expected that the basic protein isolated from the ribosomes of these bacteria will also exhibit this similarity. In order to confirm this the ultra-violet absorption curves of these proteins have been studied, and partial amino acid analyses were performed. Finally an attempt was made to determine the iso-electric points of the ribosomal proteins by studying their migration in polyacrylamide gels, and the data obtained from these experiments was also used to plot mobility-pH curves for the various protein components.

Figure 20.

Ultra-violet Absorption Curves of Basic Proteins Isolated from Bacterial Ribosomes



B. megaterium ribosomal basic protein
0.165% w/v solution
1.0 cm. light path



E. coli ribosomal basic protein
0.085% w/v solution
0.5 cm. light path

ii. Ultra-violet Absorption Curves

The ultra-violet absorption curves of the basic proteins isolated from the ribosomes of B. megaterium and E. coli are plotted in Fig. 20. These are very similar in form to the calf thymus histone curve (Fig. 8), and the presence of tyrosine, but not tryptophan, is therefore indicated in the proteins. The rather high non-specific background absorption at lower wave-lengths, which occurred with the basic proteins isolated from the whole bacteria (Fig. 16) has been greatly reduced. This may mean that purer basic protein preparations are obtained from the ribosomes than from the whole bacteria or that the light scattering by the protein molecules in solution is reduced. However, since these curves do exhibit less of this non-specific absorption, the estimations from them of the minimum tyrosine content of the proteins should be more accurate, and closer to the true values.

TABLE 5

The analysis of arginine, lysine, glutamic acid, aspartic acid, and tyrosine residues in the basic proteins isolated from the ribosomes of *E. coli* and *B. megaterium*. Also given, for comparison, the corresponding data for the total basic protein of *B. megaterium*, unfractionated calf thymus histone, and the β fraction of calf thymus histone

All values given as g. amino acid residue in 100 g. protein

Amino acid Residue	*E. coli* ribosomal basic protein	*B. megaterium* ribosomal basic protein	*B. megaterium* total basic protein*	Unfractionated calf thymus histone**	β fraction, thymus histone***
Arginine	11.1 (11.3, 10.9)	10.7 (10.7, 10.7)	11.4	10.3	16.1
Lysine	15.0 (15.0, 15.0)	14.6 (14.6, 14.6)	14.2	15.5	9.9
Glutamic acid	7.2 (7.1, 7.4)	6.6 (6.6, 6.6)	8.1	8.5	10.7
Aspartic acid	7.8 (7.5, 8.1)	8.3 (8.2, 8.5)	9.0	4.4	5.7
Tyrosine	2.9 (minimum value)	2.9 (minimum value)	1.9 (minimum value)	3.3	3.8
Arg + Lys	26.1	25.3	25.6	25.8	26.0
Glu + Asp	15.0	14.9	17.1	12.9	16.4
$\frac{\text{Arg + Lys}}{\text{Glu + Asp}}$	1.74	1.70	1.50	2.00	1.58

* See p. 69.

** Data of Crampton *et al.* (1955) converted to g. amino acid residue in 100 g. protein

*** Data of Cruft *et al.* (1958)

iii. Partial Amino Acid Analyses

The ribosomal basic proteins were hydrolysed in sealed tubes. Arginine, lysine, glutamic acid and aspartic acid in the hydrolysates were then separated by low voltage electrophoresis and estimated by the ninhydrin-copper method. The amounts of tyrosine (minimum values) in the proteins were determined from the ultra-violet absorption curves. The results of these analyses, expressed as g. amino acid residue in 100 g. protein, are given in Table 5, together with the results obtained previously for the total basic protein of B. megaterium (p. 69), and the published data for unfractionated calf thymus histone and the β histone fraction of calf thymus. It is seen that the ribosomal basic proteins of the two bacteria contain very similar amounts of the amino acids studied. They are also similar in composition to the total basic protein of B. megaterium and, apart from a rather higher content of aspartic acid, to the unfractionated calf thymus histone. These values for the ribosomal basic proteins of bacteria are therefore well within any range that could be laid down as characteristic of the vertebrate histones.

iv. Polyacrylamide Gel Electrophoresis

Cruft (1964) considers that the differences in the separation of histone components achieved with moving boundary electrophoresis and starch gel electrophoresis are not explained solely by the molecular sieving effects of starch gels. The slow migration in starch gel of the α histone fraction, and the rapid decrease in the mobility and resolution which occur if the pH of starch gels is increased above 6, indicate the presence in the starch of anionic groups (presumably carboxyls) which cause some degree of combination between the histones and the starch itself. The sharp leading edges of many bands in starch gels, and the increase in mobility which occurs with heavy loading, also support this theory.

For this reason the use of starch gels for the determination of the iso-electric points of basic proteins was avoided. Instead the electrophoresis was carried out using polyacrylamide gels. These gels contain no dissociable groups and are apparently superior to starch gels for the electrophoresis of histones (Cruft, 1964). Moreover it was considered that the absence of charged groups in these gels would ensure that they are free from undesirable electroendosmosis effects.

10% polyacrylamide gels were prepared as described by Cruft (1962). 4 g. acrylamide (Kodak, Ltd., London) and 80 mg. NN' methylene-bisacrylamide (L.Light & Co., England) were dissolved in 40 ml. buffer in a conical flask. 0.4 ml. of a 10% (v/v) solution of NNN'N' tetra methylethylene diamine (L. Light & Co., England) in ethanol and 0.4 ml. freshly prepared 10%(w/v) aqueous ammonium persulphate (B.D.H., England) were then added with gentle stirring. After briefly evacuating the flask at a water pump to remove dissolved air, the solution was poured into a perspex trough (15 cm. x 5 cm. x 0.3 cm.) and covered with a perspex lid, care being taken to exclude all air bubbles. The gels were allowed to set for at least two hr. before use.

Veronal-acetate-HCl buffers were used over the pH range 3.0 to 9.0, and for pHs above 9.0 glycine-NaOH buffers were used. These buffer systems were chosen as Cruft (1953) had found them to be suitable for the moving-boundary electrophoresis of histones.

Veronal-acetate-HCl buffer:

A 0.286M stock solution containing 19.43 g. hydrated sodium acetate and 29.43 g. sodium veronal per litre was prepared (i.e. 0.143M with respect to each of the two salts). For the preparation of the gels this buffer was diluted ten times, the particular pH required being obtained by the addition of the requisite quantity of HCl. The same dilution of buffer was used in the buffer reservoirs of the electrophoresis apparatus adjacent to the gel. For the outer reservoirs (electrode compartments) the stock solution was diluted four times.

Glycine-NaOH buffer:

A 0.286M stock solution of glycine was prepared. This was diluted ten times for the preparation of gels and for filling the buffer reservoirs adjacent to the gel, the required pH obtained by the addition of NaOH. For the outer reservoirs a four fold dilution was used.

The pH of gels was measured before electrophoresis by inserting small pieces of narrow range indicator paper into slits cut at the edge of the gels. After electrophoresis the pH was checked on a portion of the gel (about 5 g.) which was chopped up into small pieces and extracted for approximately 1 hr. with half a volume of distilled water. The pH of this mixture was then read on a pH meter.

It was usually found that the polymerisation reaction would cause the pH of gels to vary slightly from the pH of the buffer used in its preparation. To ensure that gels were at the correct pH before the protein samples were inserted, they were subjected to a preliminary run at 5v/cm. for 2 hr., thus introducing the correct pH buffer into the gel by ionophoresis. This procedure was found to be quite adequate for pH values up to 9.0. However above this the gel pH was always lower than that of the buffer used in the preparation, and even the preliminary run failed to give the correct pH adjustment. Therefore to obtain gel pHs above 9.0 the gel was removed from the trough and left to diffuse overnight against 2 litres of buffer contained in a

stoppered cylinder. Unfortunately the pH of these gels fell again during the electrophoresis runs. Using pH 10.5 buffer, the final pH of the gel after electrophoresis was 10.0, and with pH 11.5 buffer the final gel pH was 10.6. After running the gel prepared with the pH 11.5 buffer the presence of free ammonia could be detected by smell. This indicates that the pH shifts of these gels are due to hydrolysis of the amide groups. Hence above pH 9, where this effect becomes noticeable, it has to be considered that the gels are run under conditions of decreasing pH and that free carboxyl groups are being liberated which will affect the migration of basic proteins. Polyacrylamide gel electrophoresis is therefore not ideal for the determination of iso-electric points above pH 9.

Protein samples were prepared for electrophoresis by dissolving 0.5 mg. in 0.02 ml. of the appropriate buffer and adjusting the pH of the solution (narrow range indicator paper), if necessary, by the addition of 0.1N HCl or 0.1N NaOH. The samples were applied to filter paper strips, which were then inserted into razor cut

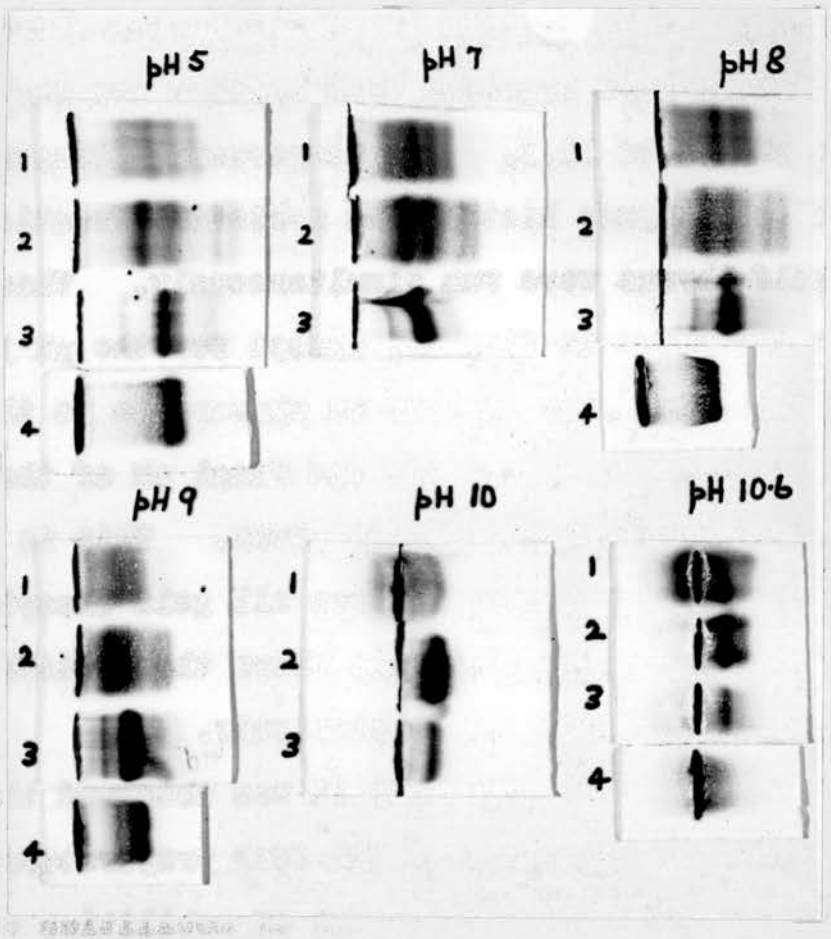
slits in the gel. The gel was covered with a thin plastic sheet and set up for electrophoresis in a similar manner to the starch gels (Fig. 2 c). The electrophoresis was carried out for 3 hr. at a potential gradient of 3v/cm. along the gel.

Polacrylamide gels were stained with amido black 10B as described for starch gels (pp. 32-33). The distances that the leading edges of the various protein bands had migrated were measured after exactly 24 hr. of destaining. For the comparison of different runs it was essential to make these mobility measurements after a constant destaining period because the gels were found to swell gradually in the destaining medium.

The gels were finally dried by immersing in ethanol. This procedure renders the gel substance white and opaque, and hence gives a good background against which to photograph the blue protein bands. The dried gels can be stored indefinitely for laboratory records.

Figure 21.

Polyacrylamide Gel Electrophoresis at Different pH's



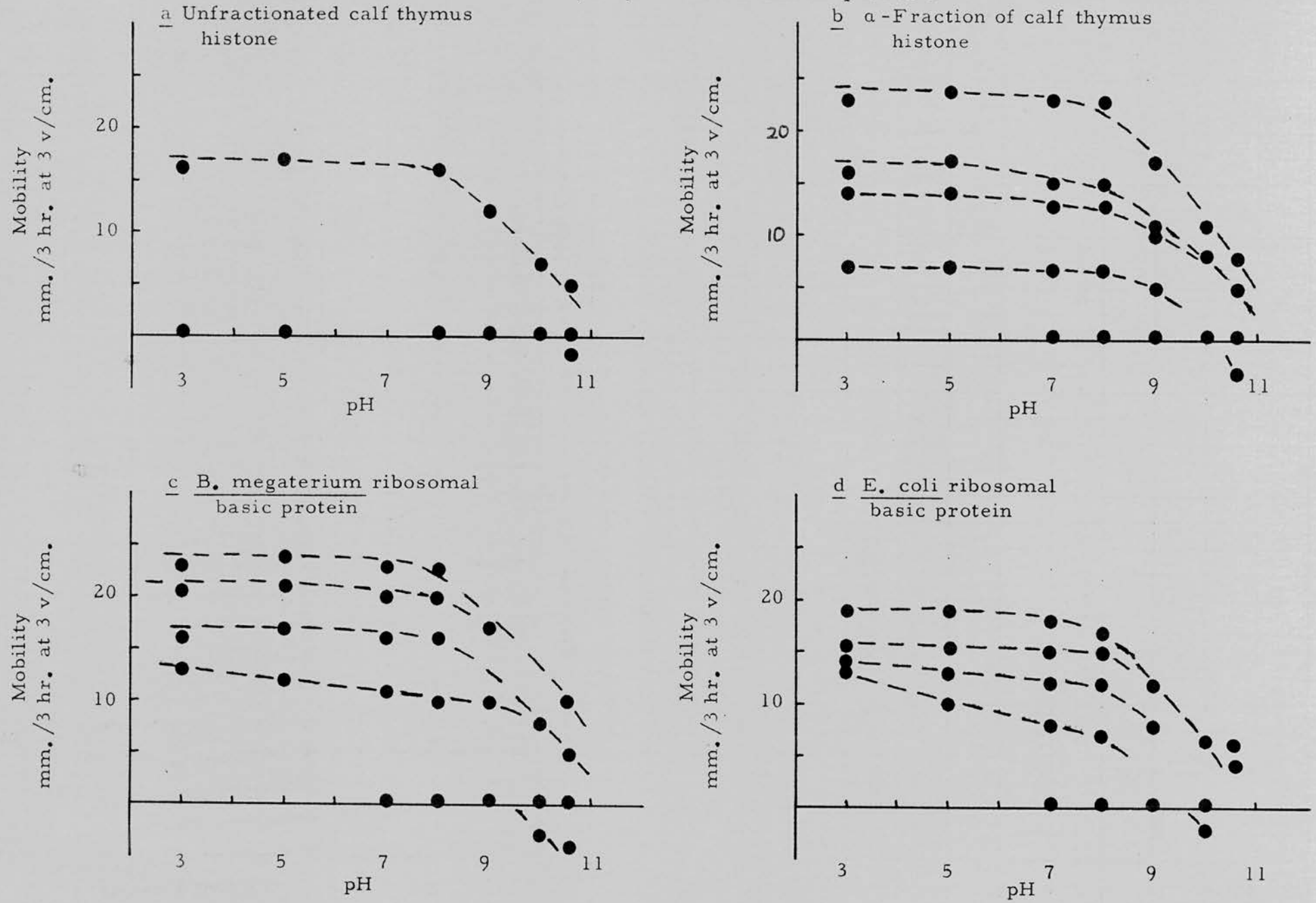
1. Basic protein isolated from E. coli ribosomes
2. Basic protein isolated from B. megaterium ribosomes
3. α fraction of calf thymus histone
4. Unfractionated calf thymus histone

The electrophoretic behaviour of the two ribosomal basic protein preparations was studied in various gels prepared with buffers ranging from pH 3.0 to 11.5. For comparison unfractionated calf thymus histone and α-histone fraction of calf thymus were run simultaneously. These gels are shown in Fig. 21, except for the pH 3.0 gel which was very similar in appearance to the pH 5.0 gel. In each case the final pH of the gel after electrophoresis is given. This is the same as the initial pH for all gels except the pH 10.0 and pH 10.6 gels where the initial pHs were 10.5 and 11.5 respectively.

From these experiments it was observed that the various components of the four preparations exhibit a fairly similar range of mobilities at the different pHs studied. Up to pH 9 none of the components of any of the preparations moved towards the anode, although above pH 7 all the samples gave an increasing amount of non-migrating aggregated material staining at the origin. The pH 10 gel showed a small portion of both the ribosomal preparations moving towards the anode. However at pH 10.6 all four preparations contained some anionic components, although much more material still migrated towards the cathode.

Figure 22.

Mobility - pH Curves of Basic Proteins
Polyacrylamide Gel Electrophoresis



No exact iso-electric point can be obtained for the components which begin to move anionically above pH 9 because of the decreasing pH of these gels. However it can be concluded from the experiments that a large portion of both the ribosomal preparations has an iso-electric point above pH 10.6, and little, if any, material has an iso-electric point below pH 10.0. The very basic nature of these proteins has therefore been confirmed.

The mobilities of the main protein bands separated by the electrophoresis are plotted against pH in Fig. 22. The spots on the pH axis represent non-migrating components which gave staining at the sample slots in the gels. The curves emphasise the similarity in electrophoretic behaviour between the basic proteins extracted from bacterial ribosomes and the vertebrate histones. Although the curves are rather difficult to draw at the higher pH values because of the poorer separation of the protein bands, they all display approximately the same shape as the mobility-pH curves for calf thymus histone obtained using moving boundary electrophoresis (Fig. 1).

V. Conclusions

The experiments on the isolation of basic proteins from whole bacteria showed that between 0.1% and 1% of the dry bacterial mass consists of basic protein. Starch gel electrophoresis, ultra-violet absorption studies, and partial amino acid analyses indicated that these bacterial basic proteins are similar in nature to the vertebrate histones.

Further experiments led to the conclusion that a large proportion of the total basic protein extracted from whole bacteria originates from the ribosomes. Bacterial ribosomes were found to contain approximately 2% of basic proteins which were shown, by partial amino acid analyses and polyacrylamide gel electrophoresis, to exhibit very similar properties to the histones. The presence of histone-like proteins in bacterial ribosomes, already indicated by the results of Waller and Harris (1961) and Zubay and Wilkins (1960) (see pp 13-14), has therefore been confirmed.

Estimations of total DNA and basic protein in the bacterial cell established that most of the DNA can not be associated with basic protein. These results are in agreement with the electron microscope observations of Van Iterson and Robinow (1961) (see p. 10), and would explain the failure of many authors to detect histones in bacterial DNA-protein preparations (pp. 8 - 10).

The possibility still exists that a small por tion of bacterial DNA could be in the form of a DNA-basic protein complex. Indeed the small amounts of the total DNA and basic protein found in the "DNA-protein" preparations could originate from such a complex. However, it is also possible that the DNA and basic protein, in both these preparations and those of Palmade (1961) (p. 12), become associated during the extraction procedure.

The significance of these results is considered more fully in the General Discussion.

THE BASIC PROTEINS OF PARAMECIUM

I. Introduction

Having established that little, if any, basic protein is associated with DNA in the bacterial cell, the results of the investigation into the basic proteins of Paramecium, which was carried out simultaneously with the earlier experiments on the bacteria, become more interesting. Unfortunately the difficulties which exist in culturing large amounts of protozoa have limited the scope of this investigation.

The material which has been studied was a gift from Professor G.H. Beale, F.R.S. This consisted of the cell debris obtained after the extraction of the cilia from Paramecium aurelia.

It was considered that the previous treatment of the paramecia would have little effect on any basic proteins present in the nuclei. The live organisms had been harvested from the culture medium by centrifugation and had then been extracted with four volumes of a salt/alcohol mixture for one hour at 2°C. The salt/alcohol mixture consisted of equal volumes of 30% (v/v) ethanol and 0.15M NaCl buffered with 0.01M

Na_2HPO_4/NaH_2PO_4 buffer pH 7.0. This extraction procedure removes the cilia from the paramecia but leaves the organisms intact. However on further centrifugation they disintegrate giving the cell debris fraction used for this investigation. The material was stored in the deep freeze until required.

Examination under the microscope of a portion of the cell debris material stained with methylene blue confirmed that it consisted mainly of broken cells with a small portion of intact paramecia. The macronuclei were visible as relatively large intensely staining particles surrounded by granular cytoplasm. The cytoplasm appeared to contain many non-staining refractile particles which were elongated, variable in size, and rather crystalline in appearance. Micronuclei could not be detected with any certainty.

II. Experimental

i. Isolation of Basic Protein from the Cell Debris of paramecia

The isolation of purified basic protein from the cell debris material was based on the general procedure used for the preparation of basic proteins from whole bacteria (pp 22-27). As with bacteria, the material was initially washed with dilute acetic acid, and then defatted and dried by treatment with ethanol and ether. The washing with dilute acetic acid resulted in some decrease in the packed volume of material, thus indicating that centrifugation at 10,000 g for 15 min. failed to recover all the smaller cytoplasmic particles.

The dry material was extracted with 0.1N sulphuric acid, but without the grinding that was used for the extraction of whole bacteria. Protein was precipitated from the extract by the addition of seven volumes of ethanol and then dried with further ethanol and ether washes. Starch gel electrophoresis showed the presence of protein migrating towards the cathode, but the staining was rather faint indicating the presence of non-protein impurities.

The basic protein was purified by dialysis and separation on a column of CM-cellulose. The amount of purified material recovered represented 1.5% of the weight of the dry defatted cell debris which had been extracted. Starch gel electrophoresis of this material gave very diffuse staining which indicated protein migrating cationically (Fig. 26, sample 2). No discrete protein bands were visible.

Basic protein is therefore apparently present in paramecia, and the isolation of nuclei from the organisms for further study was considered to be worth attempting.

ii. Preparation of Macronuclei of paramecia

An attempt was made to isolate the macronuclei from the cell debris material by differential centrifugation of a homogenate in 1% acetic acid; the technique used routinely for the preparation of calf thymus nuclei (pp. 1-2). The isolation of micronuclei was not attempted because of their small size and the difficulty in their identification.

The purification of the macronuclei was followed by examining under the microscope small portions of the material stained with methylene blue. All operations were carried out at approximately 2°C.

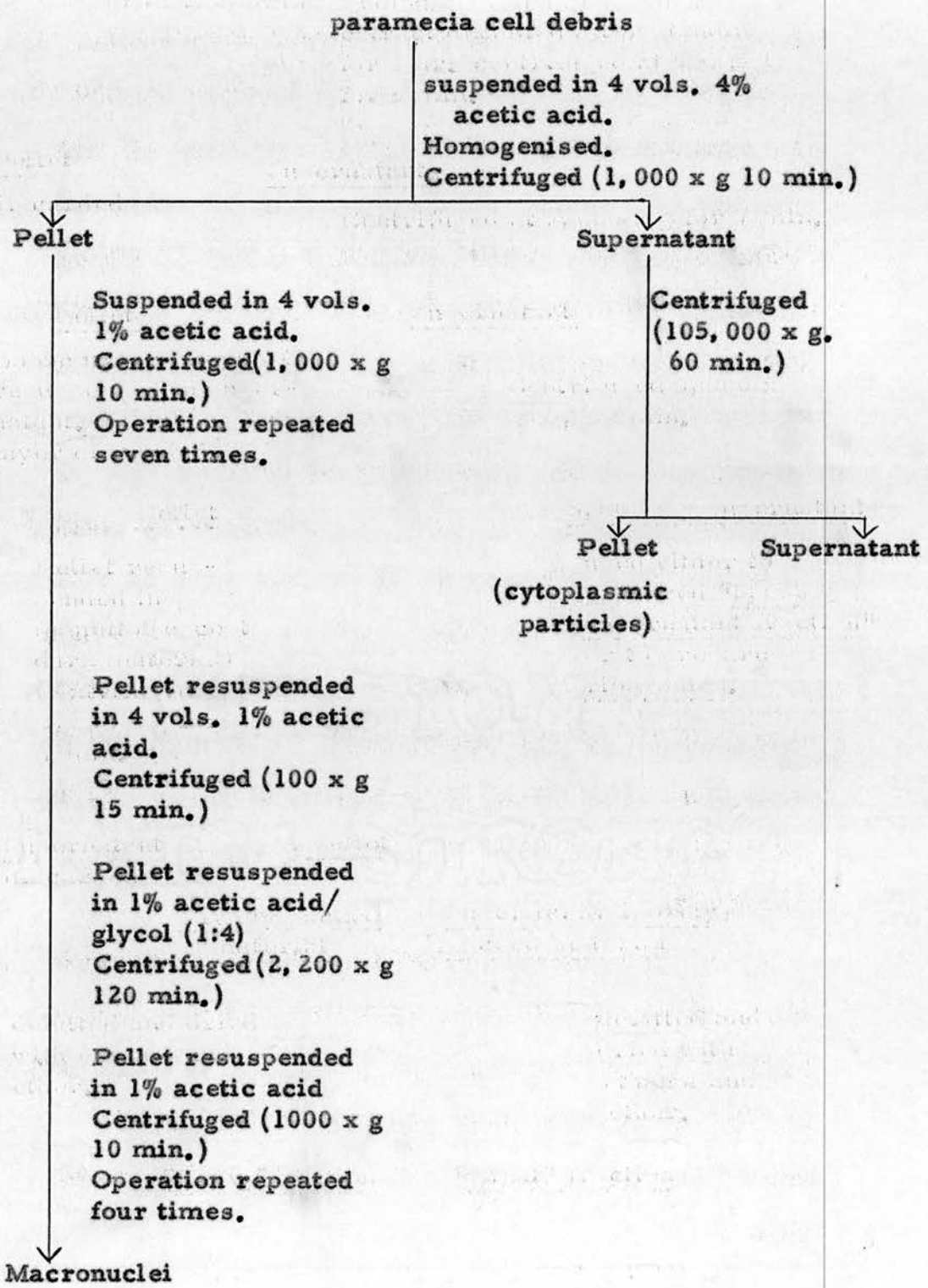
The cell debris material was first suspended in about five volumes of 4% acetic acid and homogenised gently for about 1 min. in an all glass homogeniser, the piston being rotated slowly by hand. This procedure was found to be quite adequate in removing the cytoplasmic material which had been adhering to the nuclei.

The homogenate was then centrifuged at 1,000 x g for 10 min. Microscopic examination of the supernatant showed that it contained much cytoplasmic material but none of the macronuclei. The sedimented material was resuspended in 1% acetic acid and recentrifuged at 1,000 x g for 10 min. This procedure was repeated seven times.

The final pellet after the 1% acetic acid washes was found to contain both the macronuclei and many of the elongated refractile particles. However, no other contaminating cytoplasmic material was visible under the microscope.

Figure 23.

Isolation of Macronuclei from paramecia



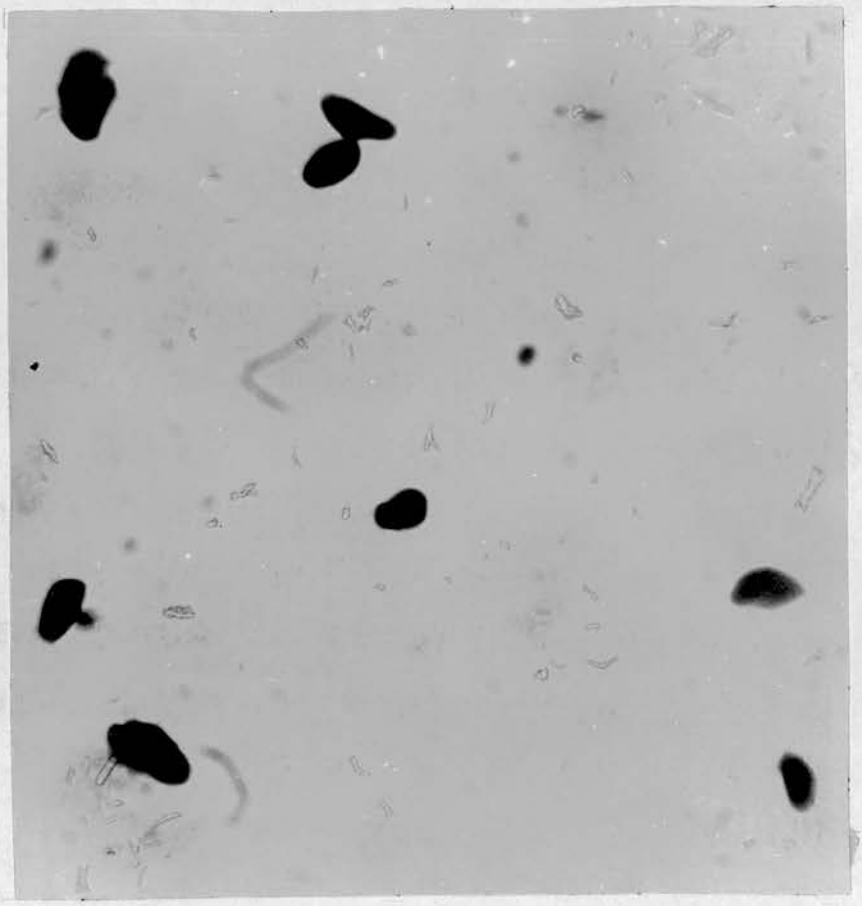
The separation of refractile particles from the macronuclei in 1% acetic acid suspensions was attempted by centrifugation at various speeds but was unsuccessful. The removal of some of the smaller refractile particles could be achieved by sedimenting the nuclei at 100 x g for 15 min., although this resulted in a few nuclei also being lost in the supernatant. After unsuccessfully trying differential centrifugation in sucrose solutions, it was eventually discovered that a further purification could be obtained by centrifugation in a mixture of 1% acetic acid (1 volume) and glycerol (4 volumes) (2,200 x g for 2 hr.).

The complete procedure adopted for a large preparation of the macronuclei is summarised in Fig. 23. The final five washes with 1% acetic acid were introduced in order to remove the glycerol.

The macronuclei obtained by this procedure were still contaminated with many of the larger refractile particles. A photograph of this preparation, stained with methylene blue and magnified approximately 500 times, is shown in Fig. 24.

Figure 24.

Macronuclei preparation from Paramecium aurelia.
Stained with methylene blue; magnification x 500.
The rod-shaped refractile particles are clearly visible.



Half the material obtained was defatted and dried by washing with ethanol and ether prior to extraction of the basic proteins. The other half was stored in the deep freeze until an attempt was made to extract DNA-protein.

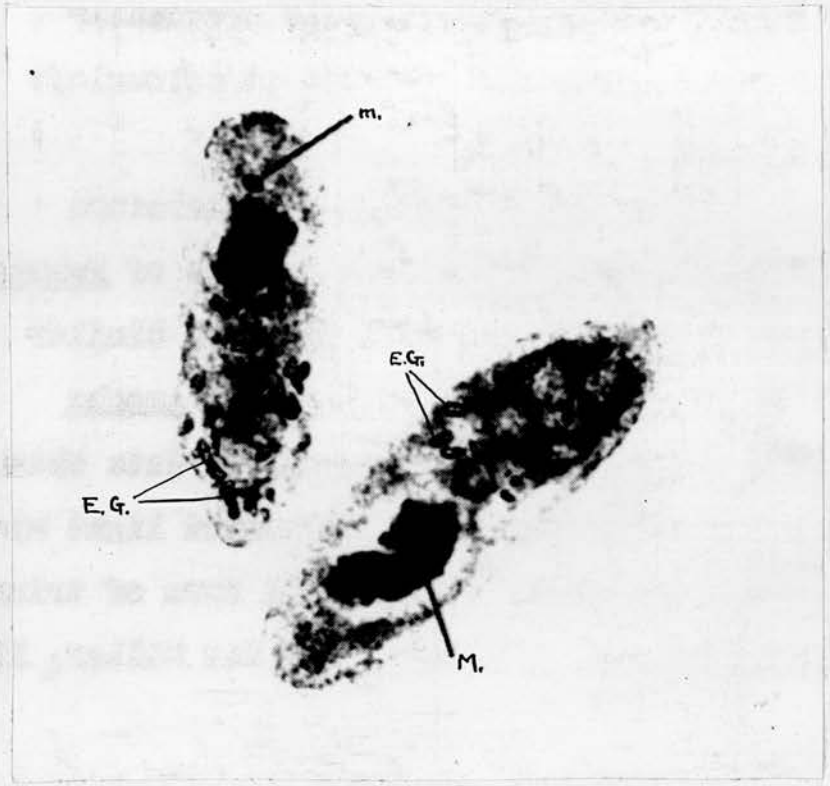
The 4% acetic acid supernatant, obtained from the first centrifugation after the homogensiation (Fig. 23) was centrifuged in the "Spinco" Model L Centrifuge at 105,000 x g for 60 min. The pellets, which would consist of the smaller particulate material of the organisms were combined and the material was dried with ethanol, and ether. The nature of this material was not studied, but it was expected to include mitochondria, ribosomes and possibly the micronuclei.

iii. Nature of the Refractile Particles Contaminating the Macronuclei

The presence of optically active crystals in the colourless form of Paramecium bursaria was reported by Wichterman (1948). However the green form of this organism, which contains symbiotic Zoochlorellae, was found to contain none of these crystals.

Figure 25.

Photographs of Paramecium caudatum, reproduced from Moses (1950).



M. - Macronucleus
m. - Micronucleus
E. G. - Excretory granules

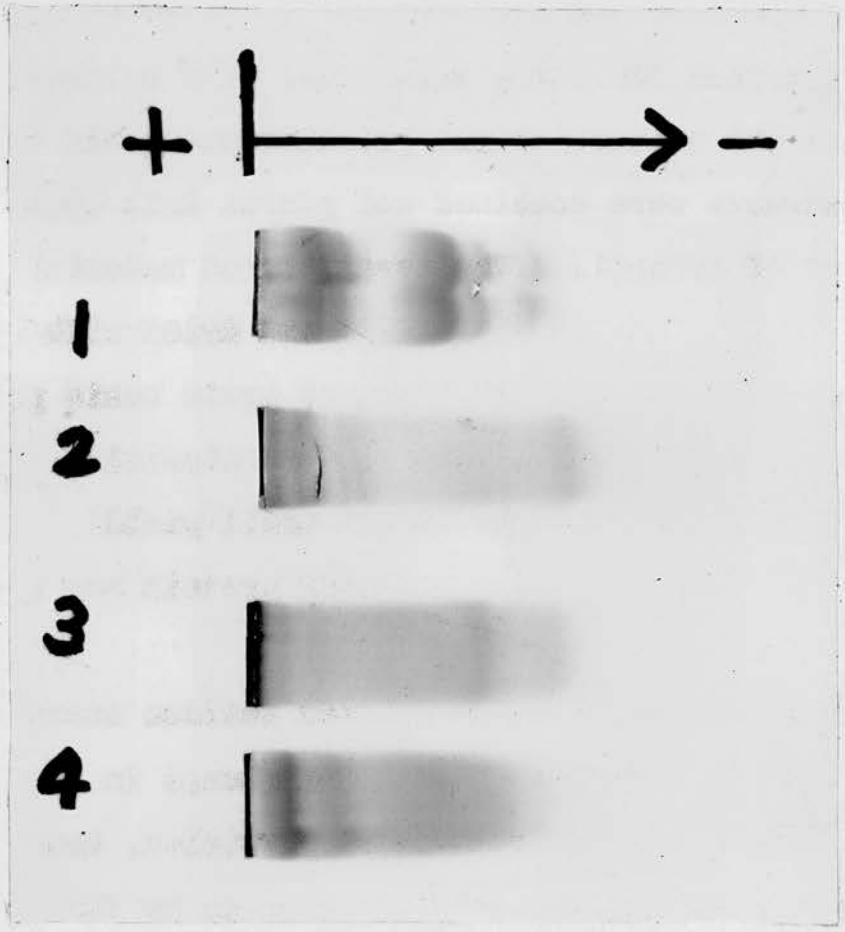
Photographs of Paramecium caudatum published by Moses (1950) show rod shaped particles occurring in the cytoplasm (Fig. 25). These were referred to as the "larger excretory granules". They appear very similar to the particles found in the cell debris material, and presumably correspond also to the crystals in colourless P. bursaria.

The refractile particles are therefore apparently present in various species of Paramecium, and it is possible that they are similar in composition to the crystals found in Amoeba proteus. These have been shown, by data obtained from X-ray diffractions and polarised light microscopy, to consist of a tetragonal form of triuret (carbonyl diurea) (Carlström and Max Møller, 1961).

Various unsuccessful attempts were made to stain the refractile particles which contaminated the present preparation of macronuclei. In particular no staining was obtained with amido black 10 B, Millon Reagent, or with ninhydrin. This indicated that the particles contained no protein, and that it could therefore be assumed that any basic protein isolated from the preparation of macronuclei orginated in the nuclei themselves and not in the contaminating particles.

Figure 26.

Starch gel electrophoresis of basic proteins isolated from Paramecium aurelia.



1. Unfractionated calf thymus histone.
2. Basic protein (fully purified) from cell debris material.
3. Basic protein (not fully purified) from macronuclei.
4. Basic protein (not fully purified) from 105,000 g. pellet.

iv. Extraction of Basic Protein from the Macronuclei

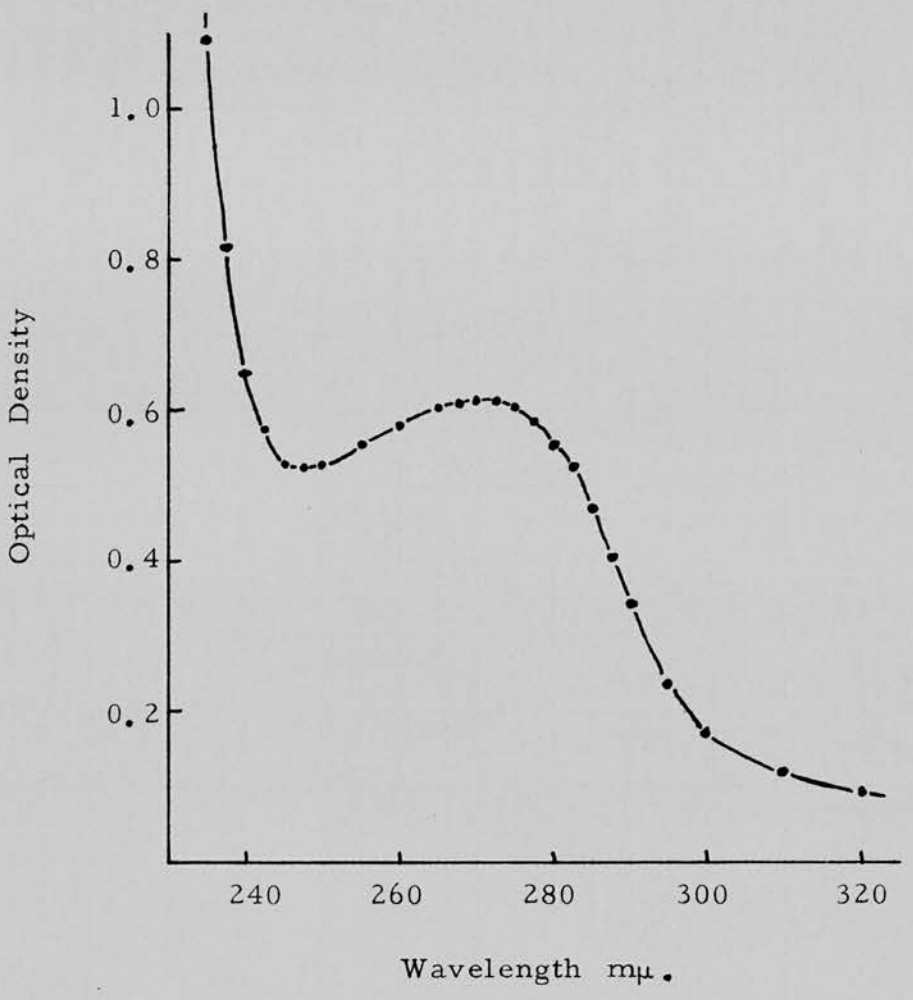
100 mg. of the dry preparation of macronuclei was extracted for 2 hr. with 8 ml. 0.1N sulphuric acid. The extraction was repeated once, and the two extracts were combined and poured into eight volumes of ethanol. The precipitated material was collected by centrifugation and dried with ethanol and ether. The yield of crude basic protein was 1.7 mg. (i.e. 1.7% of the material extracted). On account of the small yield further purification of the basic protein was not performed.

Examination of the extracted residue under the microscope showed no apparent change in the size or shape of the refractile particles, thus indicating that they were not affected by the extraction procedure.

Starch gel electrophoresis was performed on the crude basic protein, and protein migrating diffusely towards the cathode was detected (Fig. 26, sample 3). There was also heavy staining near the origin which had not been observed with the CM-cellulose purified material extracted from the complete cell debris (Fig. 26, sample 2). The heavy staining may therefore be due to non-basic protein which could have been removed by further purification.

Figure 27.

Ultra-violet Absorption Spectrum of Basic Protein Isolated from Macronuclei of Paramecium.



The ultra-violet absorption spectrum of the crude basic protein extracted from the macronuclei (0.96 mg./ 1.0 ml. 0.1N H_2SO_4; 0.5 cm. light path) is shown in Fig. 27. This does not show the typical features of the histone curve. In particular the wavelength of the absorption maximum is lower (270 mμ), and there is greatly increased absorption at the lower wavelengths. This is therefore further evidence that the basic protein is impure.

These results indicate that the maximum amount of basic protein possible in the preparation of macronuclei is 1.7% of the dry weight. However, because of the impurity of the extracted basic protein, the true percentage may be rather lower.

The maximum percentage possible for the amount of basic protein in the macronuclei themselves can not be stated accurately, as the relative mass of the contaminating refractile particles is not known. However it would appear unlikely that the basic protein in the nuclei could represent more than 2% of their mass.

Starch gel electrophoresis of the crude basic protein extracted from material sedimented at 105,000 x g in the "Spinco" Model L Centrifuge is shown in Fig. 26, sample 4. The crude basic protein represented 0.3% of the material extracted. This basic protein presumably originates from ribosomes and other cytoplasmic particles.

v. Attempt to Isolate DNA-protein from the Preparation of Macronuclei

An attempt was made to isolate DNA-histone from the preparation of macronuclei by extraction with 1.0M NaCl solution and precipitation at a NaCl concentration of 0.15M (method as described by Jordan, 1960, for "the preparation of calf thymus nucleohistone using solutions of high ionic strength"). No precipitate was obtained, although an ultra-violet absorption examination indicated that the extract contained an amount of nuclei acid equivalent to approximately 10% of the dry mass of the material that had been extracted.

III. Conclusions and Discussion

It has been shown that the macronuclei of Paramecium aurelia contain a small amount of basic protein. This represents probably less than 2% of the dry mass of the nuclei, and certainly less than 1.7% of the impure preparation of macronuclei which was examined. This preparation was contaminated with cytoplasmic refractile particles.

Moses (1950) has shown by quantitative cytological analyses that both the macronuclei of Paramecium caudatum contain remarkably little DNA. DNA was found to represent approximately 6%, and RNA approximately 9% of the total nuclei acid and protein present in the nuclei. Moses also concluded, in agreement with the above results, that little, if any, histone is present in the nuclei.

If it is assumed that the macronuclei of P. aurelia also contain approximately 6% DNA, then only a small portion of the DNA could be in the form of a typical nucleohistone complex. Further evidence for the presence of little nucleohistone in these macronuclei is given by the failure of the attempt to isolate DNA-histone from them.

The possibility that the small amount of basic protein found in the macronuclei is combined with DNA still exists. However some or all of this protein could be associated with the nuclear RNA, or it could be completely free from any association with either of the nuclei acids.

THE BASIC PROTEINS OF NEUROSPORA CRASSA

I. Introduction

The mould Neurospora crassa(N. crassa) was considered to be a particularly suitable micro-organisms for the present investigation as it can be readily grown in large quantities in a relatively simple culture medium. Apart from various inorganic salts, the only nutritional requirements of the organism are a simple sugar (as carbon and energy source) and biotin. Moreover N. crassa has been widely used for studies in the field of biochemical genetics (Fincham and Day, 1963). If typical nucleohistones occur in the cell nuclei of N. crassa, then this organism might prove satisfactory for investigations into the metabolism and function of histones.

Method of culturing the mycelia of Neurospora crassa

The mycelia of N. crassa (St. Laurence, stain A) were grown in the minimal nitrogen medium of Vogel (1956). A stock solution, of fifty times the culture concentration, was prepared containing the inorganic salts and biotin. This was stored at 2°C and was kept sterile by the addition of a few drops of chloroform.

30 ml. of stock medium and 30 g. of sucrose were made up to 1.5 l. in a 2 l. conical flask. The flask was plugged with non-absorbant cotton wool and sterilised in an autoclave for 15 min. at 15 lbs./in.^{2}. After cooling the medium was inoculated with conidia suspended in sterile water to give a concentration of approximately 1 x 10^6 condidia per ml. of medium. The flask was then placed in an incubator at 25°C for 40 hr. Constant mixing and aeration were achieved by bubbling air from the compressed air line through the medium. The air was cleaned and sterilised by passing it through a large sterile cotton wool pad.

The mycelia were harvested by filtering on a Buchner funnel and were washed with large volumes of distilled water. If the organisms were not to be used immediately the mycelial "mat" was chopped up with scissors and either stored in the deep freeze or freeze dried.

The large quantities of conidia used for inoculating the medium were produced by growing N. crassa on agar slants in 500 ml. flat medical bottles. 1 ml. of stock medium, 0.5 g. sucrose, 1 g. agar and 50 ml. of water were measured into the bottles, which were then plugged with cotton wool and sterilised (15 min. at 15 lbs./in.^{2}). The bottles were placed horizontally to cool, thus producing an even layer of agar gel over one of the inside flat surfaces. A small inoculum of conidia was placed on the agar and the bottles were incubated for 3 days at 25° C. After removal from the incubator the bottles were kept upright in daylight at room temperature. During four or five days large quantites of conidia were formed. These were washed out of the bottles with sterile water and the suspension was filtered through sterile cotton wool. The concentration of conidia in a drop of the suspension was determined by counting the conidia under a microscope.

II. Experimental

i. Isolation of Basic Proteins from Whole Mycelia

2 g. of freeze dried mycelia were broken up into a coarse powder by grinding in a mortar. The material was then defatted by extracting it three times with ethanol. After three further washes with ether the material was "redried" by allowing the ether to evaporate.

The defatted mycelia were returned to the mortar and mixed with an approximately equal volume of glass powder. This mixture was ground vigorously in the presence of 0.1N sulphuric acid for 20 min. After the grinding, more acid was added to bring the total used to 50 ml., and the extraction was continued for a further 20 min. with occasional stirring. After centrifugation the residue was re-extracted for 20 min. with another 30 ml. of acid. The two supernatants were combined and poured into seven volumes of ethanol. The precipitated material was collected by centrifugation and dried in the usual manner with ethanol and ether.

The basic protein in the material extracted from the mycelia was purified by dialysis and separation on a column of CM-cellulose, using the methods as described for the isolation of bacterial basic proteins (pp.24-27). A yield of 8 mg. of the purified basic protein was obtained, indicating that the mycelia of N. crassa contain at least 0.4% basic protein.

Starch gel electrophoresis of this material confirmed that it consisted of proteins which migrate towards the cathode at pH 6. The components with the greatest mobilities migrated slightly further than those of the unfractionated calf thymus histone run simultaneously. Staining, as in the case of the basic protein extracted from paramecia, was rather diffuse.

This experiment shows that the whole mycelia of N. crassa contain basic protein, and indicates therefore that attempts to isolate basic proteins from sub-cellular components of the cell would not necessarily prove to be unsuccessful.

ii. Preparation of Nuclei

Attempts to prepare nuclei from the mycelia of N. crassa were based on the procedure of Reich and Tsuda (1961) which involves the separation of the cellular components by differential centrifugation in mannitol solutions.

Method of Reich and Tsuda (1961)

Mycelia are harvested after growing for 60 hr., washed with water, squeezed dry and washed with 0.35M mannitol. After drying the material with blotting paper, it is chopped into small fragments, cooled to 4°C, and ground in a mortar at this temperature with three times its weight of sand. When a homogeneous mass has been obtained by persistent gentle grinding, three volumes of the 0.35M mannitol solution are added and the paste is stirred until smooth. The mixture is then filtered through three layers of cheese-cloth lining a large filter funnel for about 30 min. This gives an opaque orange fluid which is almost completely free from mycelial fragments and sand.

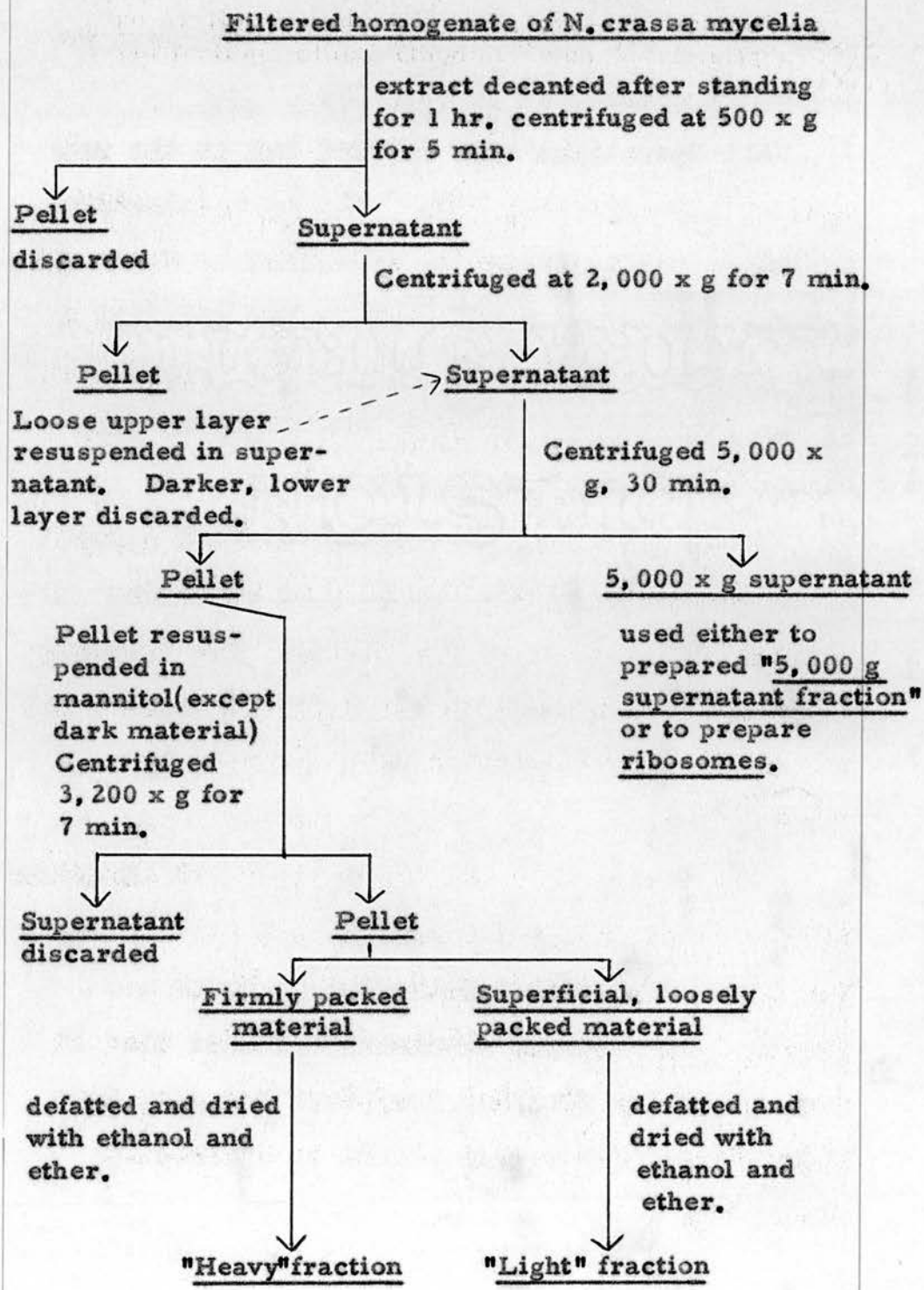
The homogenate is centrifuged at 500 x g for 5 min. The pellet has an opaque, gelatinous central portion which is resuspended in its own supernatant, and an outer white ring, which is discarded.

The supernatant is then centrifuged at 2,000 x g for 7 min. A larger pellet with a similar appearance is obtained. The central and superficial portions are resuspended in mannitol, and are combined with the pellet obtained on centrifuging the supernatant for a further 30 min. at 5,000 x g. The resultant suspension is rich in nuclei, most of which (about 85%) can be sedimented in relatively pure form by centrifugation at 3,200 x g for 7 min.

The final pellet obtained by Reich and Tsuda contained 54% of the total DNA in the filtered extract. Unfortunately the content of DNA and RNA in the dry mass of the pellet was not determined. The pellet, on staining with crystal violet, was said to show small, spherical nuclei (approximately 1μ in diameter) clustered in grape-like bunches. Sections of the pellet examined under the electron microscope showed the presence of some small contaminating particles of sand. The pellet was also seen to contain small amounts of granular cytoplasm, and an occasional mitochondrion and hexagonal crystal body.

Figure 28.

Preparation of Nuclei from N. crassa Mycelia by a Modification of the method of Reich and Tsuda (1961)



In an attempt to isolate nuclei by the method of Reich and Tsuda several changes in the procedure were introduced as a result of observations made during the experiments. This modified procedure is summarised in Fig. 28.

All operations were carried out in the cold room at approximately 2°C. The homogenisation procedure was performed as described by Reich and Tsuda. Coarse sand was used in the grinding as this gave good disintegration of the mycelia, and it was considered that fine sand might be more liable to disrupt the nuclei.

After filtering the extract through cheesecloth it was found that small sand particles settled at the foot of the flask. The filtrate was therefore allowed to stand for at least 1 hr. and then it was separated from the sand by decantation.

The pellet obtained after the first centrifugation (500 x g for 5 min.) did not exhibit the two distinct portions described by Reich and Tsuda. Microscopic examination showed that it contained many mycelial fragments and also some fine sand. The entire pellet was therefore discarded.

The pellet from the second centrifugation (2,000 x g for 7 min.) consisted of a loosely packed buff coloured upper layer covering a darker lower layer. The "outer, white ring" described by Reich and Tsuda was again not observed. The lower layer still contained some mycelial fragments and sand, and was therefore discarded. The superficial layers of the pellet were resuspended in the supernatant which was then centrifuged at 5,000 x g for 30 min. A small amount of material at the base of the pellet was again rejected. The remainder of the pellet, which consisted of firmly packed material covered by a loose superficial layer, was resuspended in mannitol. The supernatant from the 5,000 x g centrifugation was poured into seven volumes of ethanol. The precipitated material ("5,000 g. supernatant fraction") was washed three times with 50% (v/v) aqueous ethanol in order to remove some mannitol which had been precipitated. It was then dried with ethanol and ether, and was kept for the analysis of DNA and RNA.

After the final centrifugation at 3,200 x g for 7 min., the firmly packed and loosely packed layers were separated in order that they could be examined individually. The former material has been termed the "heavy fraction" and the latter the "light fraction". Small samples of both fractions were suspended in 1% acetic acid and kept for microscopic examination.

Both fractions were then extracted four times with 50% (v/v) aqueous ethanol to remove the mannitol, and were finally defatted and dried by the usual procedure of ethanol and ether washes.

From 30 g. of mannitol washed mycelia (weighed after squeezing dry between blotting paper) 11 mg. of the "heavy fraction" and 32 mg. of the "light fraction" were obtained. The DNA and RNA in these two fractions, and in the 5,000 g supernatant material, were determined by the diphenylamine and orcinol methods respectively. The results are summarised in Table 6 .

TABLE 6

Estimations of DNA and RNA on fractions obtained from homogenate of *N. crassa* mycelia.

Fraction	Dry Weight	% DNA	% RNA	Total DNA in fraction	Total RNA in fraction
"Heavy"	11mg.	3.2	4.1	0.4 mg.	0.5 mg.
"Light"	32mg.	6.0	2.8	1.9 mg.	0.9 mg.
5,000 g supernatant	1490mg.	0.3	3.0	5.2 mg.	45.3 mg.

These results indicate that the "light fraction" is probably a purer preparation of nuclei than the "heavy fraction" as it contains a higher percentage of DNA and a lower percentage of RNA. The 5,000 g supernatant contains more than twice the amount of DNA contained in the entire final pellet ("light" and "heavy" fractions). As some DNA is probably lost in the final supernatant (centrifugation at 3,200 x g for 7 min.) the recovery in the pellet of the DNA originally present in the filtered extract will only be about 30% or rather less. This is considerably lower than the 54% recovery claimed by Reich and Tsuda.

Microscopic examination of small drops of the 1% acetic acid suspensions of the "heavy" and "light" fractions showed the presence in both of small, apparently spherical, particles which

exhibited Brownian motion. Even the largest particles were only about one tenth of the diameter of nuclei prepared from calf thymus gland. Both fractions were very similar in appearance, although the average size of the particles in the "heavy fraction" was possibly slightly greater.

For further examination drops of the 1% acetic acid suspensions were taken to dryness on microscope slides, and the deposited material was fixed with formalin/acetic acid/ethanol (10:5:85 by volume). Staining with crystal violet showed that the particles had aggregated into clumps, possibly corresponding to the grape-like bunches of Reich and Tsuda. This aggregated material also stained with the Feulgen stain (10 min. acid hydrolysis and 30 min. staining time), but the intensity of the pink colour was weak compared to that obtained with thymus nuclei. Even under the oil immersion lens it was not possible to distinguish between Feulgen stained nuclei and other cellular particles, and it was concluded that the light microscope possesses insufficient resolving power to determine the purity of N. crassa nuclei. This would require the examination of preparations under the electron microscope. However, assuming

that DNA is contained exclusively in the nuclei, the purification of nuclei could be followed by observing the increase in the DNA content of material under preparation.

An attempt was made to improve the purification of the nuclei by extending the differential centrifugation process. The method was repeated as already described until after the centrifugation at 5,000 x g for 30 min. The pellet from this centrifugation was resuspended in mannitol, and the suspension was then resubjected to the differential centrifugations that had already been performed (i.e. centrifugations at 500 x g for 5 min., 2,000 x g for 7 min., and 5,000 x g for 30 min. were repeated, and the various pellets and supernatants were treated as before). The pellet after the second centrifugation at 5,000 x g was then recycled once more through this additional sequence of operations. The final 5,000 x g pellet was resuspended in mannitol and centrifuged at 3,200 x g for 7 min. The pellet was separated as before into "heavy" and "light" fractions which were dried, weighed, and analysed for DNA and RNA.

From 30 g. of the mannitol washed mycelia, 5.5 mg. of the "heavy" fraction and 13.7 mg. of the "light" fraction were obtained. As would be expected the additional operations have resulted in a decreased yield of nuclei. The analyses showed that the "heavy" fraction contained 1.9% DNA and 2.8% RNA, and the "light" fraction contained 6.1% DNA and 3.2% RNA. Thus on the basis of DNA content the purity of the nuclei has not been improved.

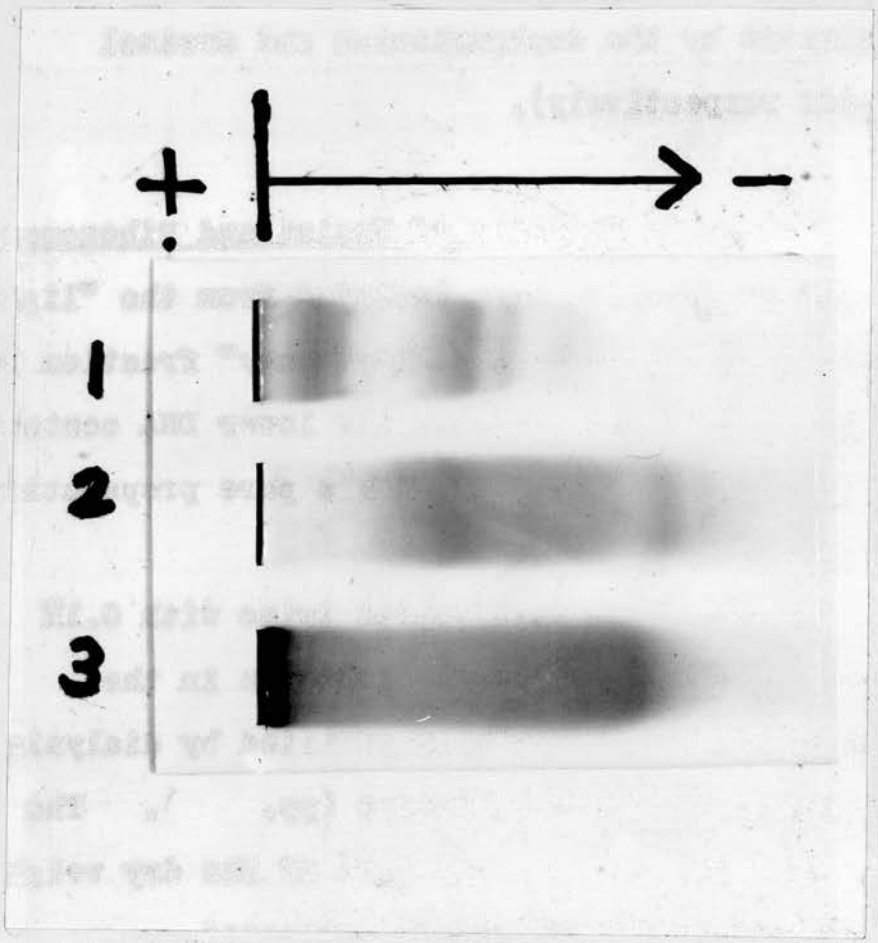
It was therefore concluded that the original procedure is more suitable as it involves less operations and appears to give higher yields of material.

iii. Isolation of Ribosomes

The ribosomes of N. crassa mycelia were isolated from the 5,000 x g supernatant obtained during a preparation of the nuclei. This supernatant was centrifuged twice at 30,000 x g for 30 min. in the M.S.E. "17,000" Refrigerated Centrifuge, in order to remove the mitochondria and any other cell particles larger than the ribosomes. The ribosomes were then sedimented from the supernatant by centrifugation in the "Spinco" Model L Centrifuge (Rotor No. 40) at

Figure 29.

Starch gel electrophoresis of basic proteins isolated from Neurospora crassa



1. Unfractionated calf thymus histone.
2. Basic protein isolated from nuclear preparation.
3. Basic protein isolated from ribosomal preparation.

145,000 x g for 120 min. The pellet was dried with ethanol and ether. This ribosomal preparation was found to contain 0.1% DNA and 17% RNA (estimated by the diphenylamine and orcinol methods respectively).

iv. Basic Proteins of Nuclei and Ribosomes

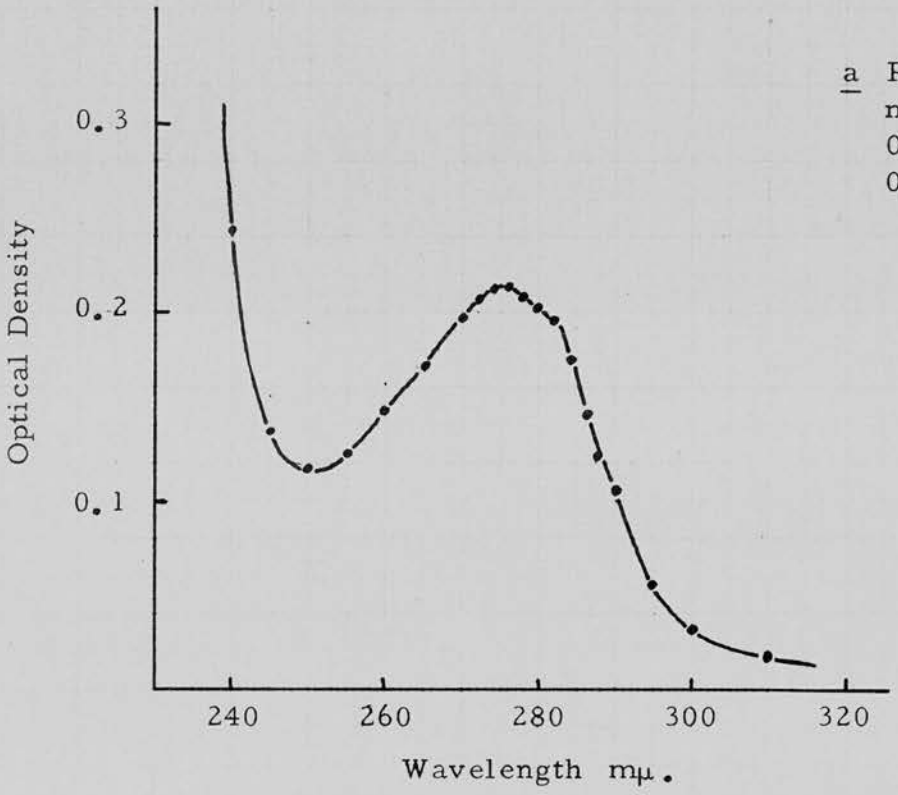
Basic proteins were isolated from the "light" fraction of the nuclei. The "heavy" fraction was not studied further because the lower DNA content indicated that it was not such a pure preparation of nuclei.

The nuclei were extracted twice with 0.1N sulphuric acid. The basic proteins in the combined extracts were then purified by dialysis and separation on CM-cellulose (pp.24-27). The yield of basic protein was 1.6% of the dry weight of the preparation of nuclei extracted.

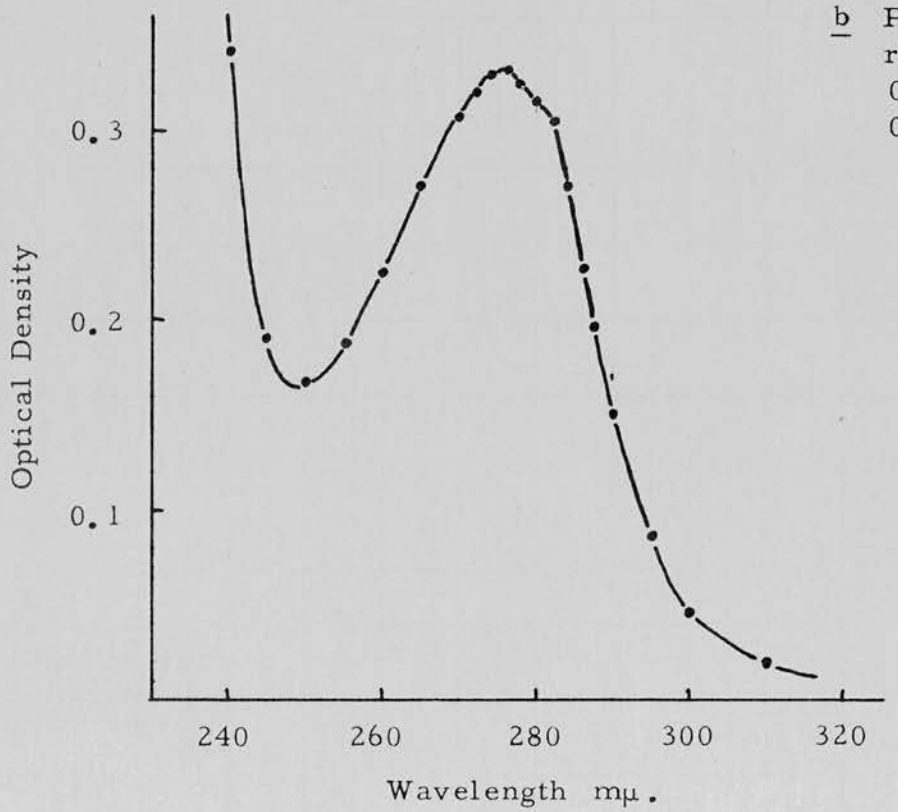
The basic proteins in the acid extracts of the ribosomal preparation were similarly purified. The basic protein obtained represented 3.0% of the dry weight of the ribosomes extracted.

Starch gel electrophoresis of both basic protein preparations was performed (Fig. 29). The material from the nuclei gave some six bands

Figure 30. Ultra-violet Absorption Curves of Basic Proteins Isolated from Neurospora crassa



a Protein isolated from nuclear preparation. 0.104% w/v solution 0.5 cm. light path



b Protein isolated from ribosomal preparation 0.115% w/v solution 0.5 cm. light path

migrating towards the cathode and all with fairly high mobilities (sample 2). There was little staining at or near the origin. On the other hand the ribosomal basic protein gave very diffuse staining which was particularly heavy close to the origin (sample 3).

The ultra-violet absorption spectra of both preparations are shown in Fig. 30. These are very similar to the typical histone curve (Fig.8), and hence the presence of tyrosine, but not tryptophan, is indicated in both preparations.

v. Heterogeneity of the Preparation of N. crassa Nuclei

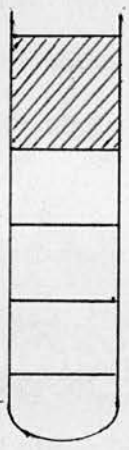
In order to determine if the "light" fraction of the nuclei prepared from N. crassa mycelia was homogeneous, a suspension of the material was subjected to centrifugation through a "stepped" concentration gradient of sucrose.

Two 5.5 ml. "lusteroid" tubes for the SW39 "swing-out" Rotor of the "Spinco" Model L Centrifuge were filled as shown in Fig. 31 a. Each layer of mannitol/sucrose solution was pipetted onto the surface of the previous layer, with great care being taken to avoid undue mixing at the

Figure 31.

Stepped Density Gradient Centrifugation of N. crassa Nuclei ("light" fraction).

a. Arrangement of layers before centrifugation.



Vol. of layer	Composition of layer
1.5 ml.	"Light" nuclei suspended in 0.35M mannitol/1.0M sucrose
1.0 ml.	0.35M mannitol/1.3M sucrose
1.0 ml.	0.35M mannitol/1.6M sucrose
1.0 ml.	0.35M mannitol/1.8M sucrose
0.6 ml.	0.35M mannitol/2.0M sucrose

b. Appearance after centrifugation and analysis of separated bands.

Band No.	Weight of material	% DNA	% RNA	% Acid soluble protein
1	6.9 mg.	2.7	3.9	7
2	8.3 mg.	4.4	4.2	7
3	10.3 mg.	9.3	4.7	10
4				
5	4.7 mg.	6.9	5.5	8

interfaces. This operation was performed at room temperature as at lower temperatures the viscosity of the sucrose solutions becomes too great to allow pipetting. After leaving the tubes to cool in the cold room for 1 hr. they were centrifuged in the SW39 rotor for 90 min. at 35,000 r.p.m. (135,000 x g).

After the centrifugation the tubes were removed carefully from the rotor buckets and kept vertical. The preparation of nuclei had separated into bands which occurred at the "steps" in the sucrose concentration gradient (Fig. 31 b). The five bands were collected separately by removing them in sequence with a pipette and were transferred to weighed centrifuge tubes. Bands 3 and 4 were combined giving four fractions in all for analysis. The material in each centrifuge tube was washed three times with 50% ethanol to remove the mannitol and sucrose, and was then dried with ethanol and ether. After weighing the four fractions, the acid soluble proteins were extracted with 0.1$\bar{N}$ sulphuric acid, and the DNA and RNA in the residues were estimated. The acid soluble proteins were precipitated with ethanol, dried with ethanol and ether, and weighed.

Insufficient of the material was obtained to allow further purification of the basic protein on CM-cellulose.

The distribution of the preparation of nuclei into bands at the interfaces of the different mannitol/sucrose solutions indicates that the preparation is not homogeneous. The percentages of DNA and RNA contained in each fraction vary considerably thus confirming this heterogeneity. The high percentage of DNA in bands 3 and 4 suggests that they contain the greatest proportion of nuclei. However the presence of DNA in all the fractions shows that there must either be some variation in the density of DNA-containing particles (nuclei?) or that aggregation between different particles has occurred.

It was found that the acid soluble protein extracted from the material present in bands 3 and 4 (the best preparation of nuclei on the basis of DNA content) behaved very like the basic protein from the "light" fraction of nuclei when subjected to starch gel electrophoresis (Fig. 29, sample 2).

III. Conclusions and Discussion

Using a modification of the method of Reich and Tsuda (1961) a preparation of nuclei containing approximately 6% DNA, 3% RNA and 1.6% basic protein was obtained from N. crassa mycelia. On the basis of starch gel electrophoresis, ultraviolet absorption, and its combination with CM-cellulose at pH 7, the basic protein appears to be similar to the typical vertebrate histones. As the nuclear preparation was found to be heterogeneous, the basic proteins which were studied may not necessarily have originated entirely from nuclei. However, the analyses of the nuclear preparation do indicate that much of the N. crassa DNA can not be in the form of a typical nucleohistone complex. As with the bacteria and paramecia already studied, the possibilities still exist that either the DNA could be entirely free from combination with basic protein or that a small portion could be complexed.

"Stepped" concentration gradient centrifugation, which was used to demonstrate the heterogeneity of the nuclear preparation, might provide a suitable method for further purification of N. crassa nuclei. With a larger capacity "swing-out" rotor it should be possible to obtain

sufficient pure nuclei to enable a full study of their basic proteins.

Difficulty in preparing nuclei from N. crassa mycelia by the method of Reich and Tsuda has also been experienced by Baer (1964) working in California, who found upon microscopic examination that no more than 10% of the pellet consisted of nuclei. Recently she has improved the preparation by freezing the mycelia with liquid nitrogen and homogenising in a Waring Blender. The proteins extracted with acid from the nuclei were subjected to starch gel electrophoresis at pH 4.1 in the presence of urea. At least 10 bands were obtained, several with greater mobility than any of the calf thymus histone components.

Although Baer has not fully characterised the nuclei or purified the basic proteins on CM-cellulose, her results are in agreement with those of this investigation.

GENERAL DISCUSSION

General Discussion

The investigation of the basic proteins in bacteria has led to the conclusion that most of the DNA in the bacterial cell must be free from combination with histone-like protein. This appears to be in agreement with those authors who were unable to detect histones in either bacterial cells or DNA-protein preparations (pp. 8-10).

However DNA-proteins, prepared from bacteria by extraction at high salt concentration, were found to contain some basic protein. Although the yields of the preparations based on the total DNA recovered were extremely low, and DNA and basic protein together accounted for a rather small percentage of the material isolated, nevertheless it is possible that these DNA-protein preparations do represent a quantitatively minor portion of the total DNA which is associated with basic histone-like protein in the bacterial cell. Such a complex could have importance in genetic regulatory mechanisms (vide infra). However it must also be considered that the DNA-protein preparations could be artefacts formed during the preparation procedure.

One might expect the formation of DNA-protein artefacts to involve a random non-specific combination between the DNA and the basic protein of the bacterial cell. This would result in the basic protein component of the artefact resembling the total basic protein of the cell. In the experiments with B. megaterium starch gel electrophoresis indicated that the basic proteins isolated from the DNA-protein and from the whole organisms were quite unlike each other (Fig. 18), thus indicating that the DNA-protein may well not be an artefact. With E. coli the starch gel electrophoresis did reveal some similarity (Fig. 19) but this was still insufficient to indicate a non-specific combination between the DNA and the total basic protein of the cell.

There is therefore some evidence for the claim by Palmade that typical DNA-histones occur in bacteria (p. 12), although if they do exist they must represent a small portion of the total DNA in the cell. The yields of the DNA-proteins isolated by this author were not stated, but it appears from the results of the present investigation that they must have been very low. It

has already been pointed out that Palmade did not actually isolate any histone, but that her conclusions were reached after the determination of the arginine and lysine contained in the DNA-protein preparations.

Although the preparations of nuclei from Paramecium aurelia and Neurospora crassa were not completely satisfactory, it could be concluded that the DNA in these organisms must also be largely free from combination with basic protein. The nuclei of both these organisms did appear to contain some basic protein and therefore, as with the bacteria, there is again the possibility that a small portion of the DNA might be in the form of a typical DNA-histone complex. However as the macronuclei of Paramecium may contain rather more RNA than DNA it is quite possible that the basic protein is associated with RNA in the nucleus rather than with DNA. As the best preparations of N. crassa nuclei also contain appreciable amounts of RNA the possible existence of an RNA-basic protein complex in the nuclei of this organism must also be considered.

Iwai (1964) has recently reported that DNA-protein prepared from the unicellular green alga, Chlorella ellipsoidea, contains histone-like protein. The acid soluble proteins extracted from the preparation were fractionated by CM-cellulose chromatography and the amino acids compositions of the fractions were determined. The yield of the DNA-protein was not stated. Iwai, however, does state that the mass ratio of the basic protein isolated by direct extraction of whole Chlorella cells to the total DNA in the cells is only between 0.25 and 0.5. When it is considered that some of the basic protein isolated directly from whole organisms might originate from ribosomes, it appears probable that a considerable part of the DNA in Chlorella is not associated with basic protein. This would be in agreement with the situation that was shown to occur in bacteria, Paramecium, and N. crassa.

The complete absence of typical nucleo-histones in all these micro-organisms might be expected if the sole function of the histones is to control intercellular differentiation in multicellular organisms as envisaged by the Stedmans (p. 16).

However bacteria do exhibit a type of intracellular differentiation in that their enzyme complement can be altered by the processes of enzyme induction and repression. Studies of the mechanism of these processes have led Jacob and Monod (1961) to conclude that the synthesis of adaptable enzymes in bacteria follows a double genetic control. There is evidence for the existence of specialised regulator genes which control protein synthesis through intermediary "repressors" that act on the structural genes, which in turn are responsible for determining the molecular organisation of the proteins. The repressors can be either inactivated or activated by certain specific metabolites, hence causing either enzyme induction or repression.

It is tempting to suggest that the repressor in the system of Jacob and Monod could be a basic protein of the histone type. Indeed, although these authors originally considered that it might consist of RNA, recent evidence suggests that the repressor is a protein (Gallant and Stapleton, 1964).

There is no reason to suppose that similar control mechanisms do not occur in multicellular organisms where they could also be responsible for the differentiation of cells. In a fully differentiated cell most of the genetic information must be suppressed, and according to the hypothesis of the Stedmans this is achieved by the gene inhibition action of the histones. This would explain why most of the DNA in such cells is associated with histone. In unicellular organisms one would expect much less of the genetic information to be suppressed at any given time, and hence if histones do act as the genetic repressors they need not necessarily be present in such easily detectable amounts.

Bonner et al. (1963) have recently obtained good evidence favouring a gene inhibition function for the histones in multicellular organisms. These authors showed the ability of chromatin, isolated from various tissues of the pea plant, to support DNA-dependent RNA synthesis by the RNA polymerase of E. coli bacteria. This chromatin-dependent RNA synthesis was coupled to a messenger RNA-dependent ribosomal protein

synthesis system also derived from E. coli. Using immunological methods the chromatin of pea cotyledons was shown to support the synthesis of pea seed reserve globulin, a protein characteristic of cotyledons. The chromatin of pea buds, which do not normally synthesise this globulin, did not support its synthesis in vitro. Hence the control of genetic activity characteristic of living cells was to some extent preserved in this in vitro system. However when the histone was removed from the pea bud chromatin, it was found that the pea bud DNA now supported the globulin synthesis. This was considered by these authors as indicating that the genetic control observed in the system had been exerted by the histone of the chromatin.

The present investigation has shown that the ribosomes of both E. coli and B. megaterium contain approximately 2% basic protein. On the basis of ultra-violet absorption spectra, amino acid compositions, and iso-electric points these proteins appear to be extremely similar to the histones of higher organisms. A preparation of ribosomes from N. crassa was also found to contain 3% basic protein.

The question of the classification of the ribosomal basic proteins is again raised by their great similarity to the histones which suggests that the term "cytoplasmic histones" might be suitable (see pp. 5-6). However, as there is still no evidence relating them metabolically or functionally with the histones it is probably more appropriate to continue calling them the "ribosomal basic proteins".

Histone-like proteins are also known to occur in the ribosomes of several multicellular organisms (see p. 5). Leslie (1961) has demonstrated that the basic proteins of ribosomes from both guinea-pig liver and human liver cells (HLM strain) exhibit ribonuclease activity, and suggests that this activity may be involved in the process of genetic expression by depolymerising certain RNA templates. The possibility still remains to be investigated that the ribosomal basic proteins of bacteria may also have this ribonuclease activity.

Although these histone-like proteins present in the ribosomes of various organisms are quantitatively very minor components of the ribosomal

structures, there is no evidence to indicate that they might not possess great qualitative importance in the mechanism of protein synthesis or its control. As already stated the small quantity of DNA-histone which may be present in bacterial cells could also be of major significance in the regulation of genetic processes. With regard to the possibility that histones may be involved in the control of cell differentiation, it would be of great interest to investigate at what stage in the evolutionary scale the DNA-histones become quantitatively important components of the cell nucleus, and to determine if there is any relationship between the amount and complexity of histone and the degree of cellular differentiation that occurs in various organisms.

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

I wish to thank Professor R.B. Fisher in whose Department these studies were undertaken and to express my gratitude to Dr. H.J. Cruft for his constant advice and criticism.

I am also indebted to Professor G.H. Beale, F.R.S., for supplying the Paramecia and to Dr. J.F. Wilkinson, Dr. A.L.S. Munro, and Dr. I.W. Sutherland for culturing the bacteria.

APPENDIX

Summary of the distribution of histones (and protamines) in living organisms

The occurrence of protamine, instead of histone, in a particular tissue is indicated by the letter P.

Where evidence is based solely on cytochemical techniques, and not by actual isolation of histone or protamine, this is indicated by the letters CYT.

A. VERTEBRATES

Tissue	Species	Reference
Bone marrow	Rabbit	8
Brain	Guinea-pig	15
	Human	15
	Ox	15
Embryo	Chicken	12
Erythrocytes	Carp	13
	Chicken	13
	Duck	13
	Frog	13
	Pike	13
	Salmon	13
	Tench	13
	Trout	13
Kidney	Mouse	4
	Ox	2
Liver	Chicken	1
	Dog	1
	Human	1
	Mouse	1, 4
	Ox	1, 2
	Pig	1
	Rabbit	1
	Rat	1
	Salmon	1

A. (contd.)

Tissue	Species	Reference
Lymphocytes (Lymphatic leukaemia)	Chicken	1
Lymphocytes (Lymphatic leukaemia)	Human	1
Mammary gland (Carcinoma)	Human	1
Myelocytes (Myeloid leukaemia)	Human	1
Ova	Trout	5
Ovary	Rabbit - CYT	11
Pancreas	Ox	10
Placenta	Human	9
Spermatozoa	Carp	13
	Chicken - P	3
	Cod	1
	Frog	13
	Herring - P	12
	Pike	13
	Sturgeon - P	6
	Trout - P	7
	Salmon - P	12
Spleen	Chicken	1
	Human	1
	Mouse	1, 4
	Ox	1
	Pig	1
	Rat	1
Testis	Guinea-pig	2
	Ox	14
	Rabbit - CYT	11
Thymus	Chicken	1
	Human	1
	Ox	1, 2

B. INVERTEBRATES

Tissue	Species	Reference
Embryo	Helix aspersa - CYT	16
Gill	Loligo opalescens (squid)	18
Ova	Helix aspersa - CYT	16
Testis	Loligo opalescens (squid)	18
Spermatozoa	Echinarachnius parma (sand dollar)	17
	Helix aspersa P CYT	16
	Loligo pealii (squid)	17
	Loligo opalescens (squid) P	18
	Starfish	17
	Grasshopper - CYT	19
Salivary gland	Drosophila - CYT	24

C. PLANTS

Various differentiated tissues	Broad bean - CYT	
	Corn - CYT	
	Lilium henryi - CYT	20
	Onion - CYT	
Sperm nuclei	Tradescantia-paludosa - CYT	20
Nut Embryo	Pinus sibirica (cedar)	21
Embryo	Pea	22
Embryo	Rice	23
Germ	Wheat	1
	Chlorella ellipsoidea	23

References

1. Cruft, Mauritzen and Stedman (1957 a).
2. Crampton, Stein and Moore (1957).
3. Daly, Mirsky and Ris (1951).
4. Davison, (1957).
5. Felix (1959).
6. Felix, Fischer and Krekels (1952).
7. Felix, Fischer and Krekels (1956).
8. H idvégi, Árky, Antoni and Várterész (1963).
9. Hirschbein and Khouvine (1957).
10. Klyszejko, De Mende and Khouvine (1964).
11. Rejniak (1963).
12. Stedman and Stedman (1944 a).
13. Vendrely (1957).
14. Vendrely, Knobloch and Vendrely (1957).
15. Wolfe and McIlwain (1961).
16. Bloch and Hew (1960).
17. Hamer (1955).
18. Bloch (1962).
19. Bloch (1963).
20. Rasch and Woodard (1959).
21. Belozerskii and Uryson (1958).
22. Bonner and Huang (1963).
23. Iwai (1964).
24. Horn and Ward (1957).

ISOLATION OF HISTONES(?) FROM *STAPHYLOCOCCUS AUREUS*

By
Dr. H. J. CRUFT
and
J. L. LEAVER

(*Reprinted from Nature, Vol.* 192, *No.* 4802, *pp.* 556–557, *November* 11, 1961)

(*Reprinted from Nature, Vol.* 192, *No.* 4802, *pp.* 556–557, *November* 11, 1961)

Isolation of Histones(?) from *Staphylococcus aureus*

ANY theories that try to give an explanation of the function of histones have had in the past to take account of the reported absence of histones from bacteria[1,2]. If histones are solely concerned with cell differentiation or 'gene suppression' in multicellular organisms, then one might expect them to be absent from bacteria and protozoa[3,4]. But if, on the other hand, they have an even more fundamental function, related to the function of deoxyribonucleic acid (DNA) in all cells, then they should also be present in unicellular organisms.

In the present investigation an attempt was made to extract basic proteins from bacteria, following a modification of the procedure routinely used in our laboratory for the isolation of histones from cell nuclei[5].

The bacteria, *Staphylococcus aureus*, were washed with 4 per cent acetic acid, and then defatted and dried by washing several times with ethanol and then with ether. This procedure denatures many of the non-basic proteins, rendering them insoluble on extraction with acid. The acid-soluble proteins were extracted by grinding the dried bacteria with glass powder in the presence of 0·1 *N* sulphuric acid, and then centrifuging. Large amounts of non-protein material extracted were removed by dialysis against 0·1 *N* sulphuric acid, and the proteins were precipitated from the dialysed extract by the addition of seven volumes of ethanol, and dried with ethanol followed by ether.

Some of the extracted material was subjected to starch-gel electrophoresis in the presence of 4 *M* urea using a modification of the technique developed by Neelin and Neelin for use with histones[6]. The gel, rolled 1 mm. thick on a glass plate and coated afterwards with paraffin wax, was prepared with 0·014 *M* acetate buffer containing the urea, giving a *p*H within the gel of 6. After migration, the gel was stained with amido black 10*B* and showed the presence of at least eight components (Fig. 1). The direction of migration of the slower components is reversed if the *p*H of the gel is increased above 6·5.

Those components which were strongly basic, like histones, with isoelectric points well above *p*H 7 were separated from the others by using carboxymethylcellulose. The protein mixture, dissolved in bicarb-

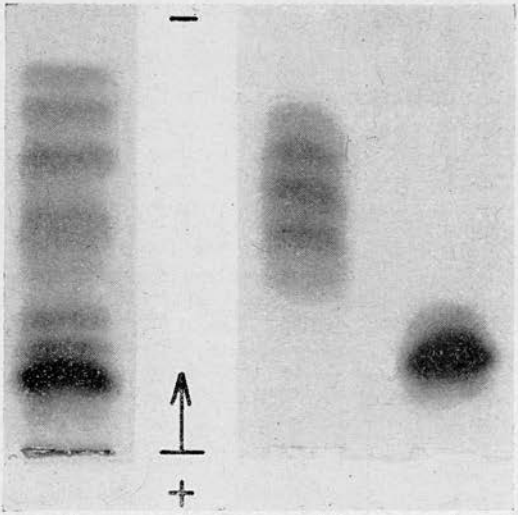


Fig. 1. Starch-gel electrophoresis of proteins extracted from *Staphylococcus aureus* with 0·1 *N* sulphuric acid : (*a*) total protein, (*b*) basic protein fraction, (*c*) non-basic fraction

onate buffer *p*H 7, was applied to a very short carboxymethyl-cellulose column previously washed with buffer. Under these conditions the non-basic components run through the column while the more basic components are retained. The column was washed with water, and then the basic proteins eluted with about 1 ml. of 0·1 *N* hydrochloric acid.

Starch-gel electrophoresis of these two fractions, recovered from the eluates by ethanol precipitation, shows the complete separation of the faster components from the remainder, which, although extracted by acid, do not have isoelectric points above *p*H 7 (Fig. 1).

Amino-acid analysis of both fractions, by acid hydrolysis followed by paper chromatography (two successive runs in one direction using an *n*-butanol, acetic acid, water system and detection of the spots by ninhydrin reagent), indicated that the faster, more basic fraction was very similar to unfractionated ox-thymus histone[7]. It contained particularly large amounts of lysine, arginine, alanine, leucine and proline with relatively small quantities of acidic amino-acids, histidine and tyrosine, while the slower, less basic fraction was quite different. It is concluded from the above results, from ultra-violet absorption and from solubility characteristics that the proteins in the faster electrophoretic fraction are very similar indeed to histones.

Thus it is clear that these bacteria contain basic proteins like the histones found in the higher forms of life. Comparable results have been obtained with *Micrococcus lysodeikticus*. These basic proteins may also occur in the DNA-protein fraction previously isolated by Vendrely *et al.* from *Escherichia coli*[8].

It is not yet possible to say whether all the basic protein components demonstrated here have come from association with the DNA or the ribonucleic acid of the bacteria. But somewhat similar components have recently been shown to occur in ribosomes prepared from *Escherichia coli*[9].

We wish to thank Dr. J. F. Wilkinson for providing us with liberal supplies of bacteria.

H. J. Cruft
J. L. Leaver

Department of Biochemistry,
University of Edinburgh.

[1] Zubay, G., and Watson, M. R., *J. Biophys. Biochem. Cytol.*, **5**, 51 (1959).

[2] Wilkins, M. H., and Zubay, G., *J. Biophys. Biochem. Cytol.*, **5**, 55 (1959).

[3] Stedman, E., and Stedman, E., *Phil. Trans. Roy. Soc.*, B, **235**, 565 1951).

[4] Leslie, I., *Nature*, **189**, 260 (1961).

[5] Cruft, H. J., Mauritzen, C. M., and Stedman, E., *Phil. Trans. Roy. Soc.*, B, **241**, 93 (1957).

[6] Neelin, J. M., and Neelin, E. M., *Canad. J. Biochem.*, **38**, 355 (1960).

[7] Cruft, H. J., Hindley, J., Mauritzen, C. M., and Stedman, E., *Nature*, **180**, 1107 (1957).

[8] Palmade, C., Chevallier, M. R., Knobloch, A., and Vendrely, R., *C.R. Acad. Sci., Paris*, **246**, 2534 (1958).

[9] Waller, J. P., and Harris, J. I., *Proc. U.S. Nat. Acad. Sci.*, **47**, 18 (1961).

Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.

REFERENCES

Ackerman, D. (1904) Hoppe-Seyl. Z. 43, 299.

Allen, R.J.L., (1940) Biochem. J. 34, 858.

Ando, T., Ishii, S. and Sato, M. (1959) J. Biochem. (Tokyo) 46, 933.

Arnon, R. and Perlmann, G.E. (1963) J. biol. Chem. 238, 653.

Baer, D. (1964) Personal Communication (Biology Dept., San Diego State College, California).

Belozerskii, A.N. (1947) Cold Spr. Harb. Symp. Quant. Biol. 12, 1.

Belozerskii, A.N. and Uryson, S.O. (1958) Biochemistry (U.S.S.R.) (English Translation) 23, 532.

Bloch, D.P. (1962) J. Histochem. Cytochem. 10, 137.

Bloch, D.P. (1963) J. Cell. Comp. Physiol. 62, Suppl. 1, 87.

Bloch, D.P. and Hew, M.Y.C. (1960) J. Biophys. Biochem. Cytol. 8, 69.

Bonner, J. and Huang, R.C. (1963) J. Mol. Biol. 6, 169.

Bonner, J., Huang, R.C. and Gilden, R.V. (1963), Proc. Nat. Acad. Sci. (Wash.) 50, 893.

Bronk, J.R. and Fisher, R.B. (1956) Biochem. J. 64, 106.

Burton, K. (1956) Biochem. J. 62, 315.

Butler, J.A.V. (1956) Radiation Res. 4, 20.

Butler, J.A.V., Cohn, P. and Simson, P. (1960) Biochim. Biophys. Acta. 38, 386.

Butler, J.A.V. and Godson, G.N. (1963) Biochem. J. 88, 176.

Carlström, D. and Max Møller, K. (1961) Exp. Cell. Res. 24, 393.

Chargaff, E. and Saidel, H.F. (1949) J. biol. Chem. 177, 417.

Crampton, C.F., Moore, S. and Stein, W.H. (1955) J. biol. Chem. 215, 787.

Crampton, C.F., Stein, W.H. and Moore S. (1957) J. biol. Chem. 225, 363.

Cruft, H.J. (1953) Ph.D. Thesis, University of Edinburgh.

Cruft, H.J. (1962) Biochem. J. 84, 47P.

Cruft, H.J. (1964) "The Nucleohistones", p.72. Ed. J. Bonner and P. T'so. Holden-Day Inc.

Cruft, H.J., Hindley, J. Mauritzen, C.M. and Stedman E. (1957 b) Nature (Lond.) 180, 1107.

Cruft, H.J. and Leaver J.L. (1961) Nature (Lond.) 192, 556.

Cruft, H.J., Mauritzen, C.M. and Stedman, E. (1957 a) Phil. Trans. Roy. Soc. B 241, 93.

Cruft, H.J., Mauritzen, C.M. and Stedman, E. (1958 a) Proc. Roy. Soc. B. 149, 21.

Cruft, H.J., Mauritzen, C.M. and Stedman, E. (1958 b) Proc. Roy. Soc. B. 149, 36.

Daly, M.M., Mirsky, A.E. and Ris, H. (1951) J. Gen. Physiol. 34, 439.

Davison, P.F. (1957) Biochem. J. 66, 703.

Dische, Z. (1955) Meth. Biochem. Anal. 2, 320.

Dische, Z. (1955) in "The Nucleic Acids" Vol. I p. 285. Eds. E. Chargaff and J.N. Davidson, Academic Press.

Evered, D.F. (1959) Biochem. Biophys. Acta. 36, 14.

Felix, K. (1959) Rapp. Cons. Chim. Solvay 11, 70.

Felix, K., Fischer, H. and Krekels, A. (1952) Hoppe-Seyl. Z. 289, 127.

Felix, K., Fischer, H. and Krekels, A. (1956) Prog. Biophys. Biophys. Chem. 6, 1.

Fincham, J.R.S. and Day, P.R. (1963) "Fungal Genetics", Blackwell Scientific Publications, Oxford.

Gallant, J. and Stapleton, R. (1964) J. Mol. Biol. 8, 431.

Hamer, D. (1955) Biol. Bull. 108, 35.

Handbook of Biological Data, 1956, ed. W.S. Spector, W.B. Saunders Co. (Philadelphia and London)

Harris, H. and Warren, F.L. (1954) Biochem. J. 57, XXXII.

Hidvégi, E.J., Árky, I., Antoni, F. and Várterész, V. (1963) Brit. J. Cancer 17, 377.

Hirschbein, L. and Khouvine, Y. (1957) C.R. Acad. Sci. (Paris) 244, 517.

Horn, E.C. and Ward, C.L. (1957) Proc. Nat. Acad. Sci. (Wash.) 43, 776.

Hutchison, W.C. and Munro, H.N. (1961) Analyst 86, 768.

Iwai, K. (1964) "The Nucleohistones", p. 59, ed. J. Bonner and P. T'so. Holden-Day Inc.

Jacob, F. and Monod, J. (1961) J. Mol. Biol. 3, 318.

Jordan, D.O. (1960) "The Chemistry of Nucleic Acids" pp. 10-12. Butterworth & Co.London.

Klyszejko, L., De Mende, M.S. and Khouvine, Y. (1964) Bull. Soc. Chim. Biol. 46, 111.

Kossel, A. (1884) Hoppe-Seyl. Z. 8, 511.

Kossel, A. (1928) "Protamine and Histone" Longmans, London.

Leslie, I. (1961) Nature (Lond.) 189, 260.

Lilienfield, L. (1894) Hoppe-Seyl. Z. 18, 473.

Lowry, O.H., Rosebrough, A.C., Farr, A.L. and Randall, R.J. (1951) J. biol. Chem. 193, 265.

Martin, S.J., England, H., Turkington, V. and Leslie, I. (1963) Biochem. J. 89, 327.

Masui, M., Iwata, T., Ishimitsu, A. and Umebayashi, Y. (1962) Biochem. Biophys. Acta. 55, 384.

Mazia, D. (1941) Cold Spr. Harb. Symp. Quant. Biol. 9, 40.

Miescher, F. (1874) Verhandl. d. naturforsch. Ges. in Basel. 6, 138. Published in "Die histochemischen und physiologischen Arbeiten von F. Miescher", Leipzig, 1897, 55.

Mirsky, A.E. (1947) Cold. Spr. Harb. Symp. Quant. Biol. 12, 143.

Mirsky, A.E. and Osawa, S. (1961) "The Cell" Vol. II, p. 677. Ed. J. Brachet and A.E. Mirsky. Academic Press.

Moore, S. and Stein, W.H. (1948) J. biol. Chem. 176, 367.

Moses, M.J. (1950) J. Morphol. 87, 493.

Muecke, P.S. (1962) Ph.D. Thesis, University of Edinburgh.

Murray, K. (1964) "The Nucleohistones" p. 15. Ed. J. Bonner and P. T'so. Holden-Day, Inc.

Neelin, J.M. and Neelin, E.M. (1960) Canad. J. Biochem. Physiol. 38, 355.

Palmade, C. (1961) Biol. Med. (Paris) 50, 129.

Palmade, C., Chevallier, M., Knobloch, A. and Vendrely, R. (1958) C.R. Acad. Sci. (Paris) 246, 2534.

Peterson, E.A. and Sober, H.A. (1956) J. Amer. Chem. Soc. 78, 751.

Phillips, D.M.P. (1962) Prog. Biophys. Biophys. Chem. 12, 211.

Rasch, E. and Woodard, J.W. (1959) J. Biophys. Biochem. Cytol. 6, 263.

Reich, E. and Tsuda, S. (1961) Biochim. Biophys. Acta. 53, 574.

Rejniak, L. (1963) Nature (Lond.) 198, 1210.

Rondle, C.J.M. and Morgan, W.T.J. (1955) Biochem. J. 61, 586.

Setterfield, G., Neelin, J.M., Neelin, E.M. and Bayley, S.T. (1960) J. Mol. Biol. 2, 416.

Smithies, O. (1955) Biochem. J. 61, 629.

Spiegelman, S., Aronson, A.I. and Fitz-James, P.C. (1958) J. Bact. 75, 102.

Stedman, E. and Stedman, E. (1944 a). Edin. Med. J. 51, 353.

Stedman, E. and Stedman, E. (1944 b). Biochem.J. 38, XXVI.

Stedman, E. and Stedman, E. (1950) Nature (Lond.) 166, 780.

Stedman, E. and Stedman, E. (1951) Phil. Trans. Roy. Soc. B. 235, 565.

Tissières, A. and Watson, J.D. (1958) Nature (Lond.) 182, 778.

Tsumita, T. and Chargaff, E. (1958) Biochem. Biophys. Acta. 29, 568.

Van Iterson, W. and Robinow, C.F. (1961) J. Biophys. Biochem. Cytol. 9, 171.

Vendrely, R. (1957) Arch. Klaus-Stift. Vererbforsch. 32, 538.

Vendrely, R., Knobloch, A. and Vendrely, C. (1957) Exp. Cell. Res. (Suppl.) 4, 279.

Vendrely, R., Knobloch-Mazen, A. and Vendrely, C. (1960) Biochem. Pharmacol. 4, 19.

Vogel, H.J. (1956) Microb. Gen. Bull. 1, 13.

Waller, J. and Harris, J.I. (1961) Proc. Nat. Acad. Sci. (Wash.) 47, 18.

Watson, J.D. and Crick, F.H.C. (1953) Nature (Lond.) 171, 737.

Wichterman, R. (1948) Anat. Rec. 101, 747.

Widra, A. (1959) J. Bact. 78, 664.

Wilkins, M.H.F. and Zubay, G. (1959) J. Biophys. Biochem. Cytol. 5, 55.

Wolfe, L.S. and McIlwain, H. (1961) Biochem. J. 78, 33.

Zubay, G. and Watson, M.R. (1959) J. Biophys. Biochem. Cytol. 5, 51.

Zubay, G. and Wilkins, M.H.F. (1960) J. Mol. Biol. 2, 105.